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An investigation of the use of otolith microchemistry to discriminate reef fish populations and assess the movement of individuals.

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An investigation of the use of otolith microchemistry to discriminate reef fish populations and assess the movement of individuals.

by

Paul M. Chittaro

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Abstract

Uncertainty about the levels of pelagic and demersal stage connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals) highlights a significant gap in our knowledge of what determines the distribution and abundance of coral reef fish. The main objective of this thesis was to assess the degree of connectivity among populations of two highly abundant Caribbean reef fish (*Stegastes partitus*, Bicolor damselfish, and *Haemulon flavolineatum*, French grunt) at specific, but different portions of their life history; pelagic and demersal stages. Specifically, I investigated the spatial scales at which pelagic larvae disperse by using recently settled *S. partitus* (Section A: pelagic stage connectivity), while adult *H. flavolineatum* from coral reefs were analysed to determine if they occupied mangrove habitats as juveniles (Section B: demersal stage connectivity). These investigations used otolith microchemistry.

The successful use of otolith microchemistry to assess connectivity requires sufficient spatial variability in chemical signatures so that natal origins can be identified, yet minimal temporal variability so that the same spatial chemical signatures can be used repeatedly to investigate movement. Therefore, in Chapter 2 (Section A) I assessed the classification of individuals to the site and time they were collected. The classification of *S. partitus* to their collection sites, separated by as little as 5 km, was moderately successful, while investigations of the temporal variability revealed substantial variability at the scale of two weeks. This spatial and temporal variability in otolith microchemistry suggested that investigations of connectivity were possible, but would require frequent recalibration of chemical signatures. Consequently, I assessed the degree to which individuals had originated from sites throughout Turneffe Atoll by comparing the otolith

edge chemistry of new recruits (which corresponds to the chemistry of the collection site) to the otolith core chemistry (which corresponds to the chemistry of the natal origin) of individuals that had been spawned at a specific time. Results indicated that 6-35% of the individuals sampled (i.e., 3-17 individuals from 48-53 analysed) had originated from one of seven sites at Turneffe Atoll, throughout the summer months in both 2002 and 2003.

Because the connectivity analysis of Chapter 2 relied upon the spatial variability in otolith chemistry, I investigated how the discrimination of populations could be improved in Chapter 3. Specifically, I examined the use of sagittal and lapillar otolith chemistry (lapillus is seldom used) to classify individuals to the sites from which they were collected. Results indicated that although sagittal and lapillar otoliths discriminated populations with similar levels of confidence (average correct classification of 81%) they did so using a different suite of elements. When the chemistries of both sagittal and lapillar otoliths were combined, the discrimination improved such that the average correct classification was 94%. Therefore, using the chemistry of both otoliths greatly improved the confidence with which fish were assigned to the sites from which they were collected. This refinement in the ability to discriminate populations has important implications for fisheries science, particularly in situations where differences among sites of interest are relatively small.

An assumption of the connectivity analysis used in Chapter 2 was that otolith elemental concentrations did not differ between fish of different life stages (i.e., larval/pelagic stage with its core chemistry versus juvenile/demersal stage with its edge chemistry). In Chapter 4, I assessed whether there was ontogenetic variability in otolith microchemistry by comparing the otolith chemistry of pre-hatch embryos to that of post-

settlement juveniles collected at the same site and time. Results indicated that elemental concentrations of embryo otoliths were between 2 and 325 times greater than that of juvenile edge chemistry (and 2 to 94 times greater than water chemistry) for Mn, Zn, Ba, Ce, and Pb. Since the environment was not the primary source contributing to the embryo chemistry, alternative causes were discussed. Regardless of the mechanism determining elemental concentrations in embryo otoliths, caution is warranted when interpreting the environmental patterns inferred from otolith cores, particularly when using them as a proxy for natal environments.

In Chapter 5 of Section B, I focused on the demersal stage of reef fish and whether otolith microchemistry could be used to discriminate *H. flavolineatum* caged in adjacent mangrove and coral reef sites in Belize and Bahamas. Significant variability in otolith trace elemental chemistry was detected among sites and habitats, which resulted in the classification of individuals separated by as little as 0.25 km (average correct classifications was between 68% and 85%). Substantial temporal variability in otolith chemistry of *H. flavolineatum* from Belize was detected, such that only 42% of fish, on average, from 2002 were correctly classified to their captive sites using chemical information from 2001. To determine whether *H. flavolineatum* collected from coral reefs in 2002 had occupied a mangrove as juveniles, their otolith chemistry deposited as juveniles was compared to the chemical signatures of mangroves and reefs in 2001. Results indicated that 36% of the sampled reef fish had juvenile otolith chemistries similar to that of a mangrove and thus, for the first time, provided evidence that mangroves contribute individuals to adult reef populations.

In Chapter 6, I expanded the sampling of *H. flavolineatum* (19 sites throughout Tumeffe Atoll; 9 mangrove and 11 reef sites, separated by 0.8 to 20m kms) to assess the extent to which individuals could be correctly assigned to the sites from which they were collected when natural movements were permitted (i.e., in the absence of cages, see Chapter 5). Results indicated that there was sufficient variability in otolith chemistry to differentiate among those individuals that were collected from a mangrove and those from a reef (average correct classification of 74% and 79%, respectively), yet enough overlap to prevent the correct classification of individuals to specific sites. As a result, the minimum scale at which fish could be discriminated, and thus movement quantified, was at the level of habitat (i.e., mangrove and coral reef).

In Chapter 7, I discuss the findings of each of these chapters in the context of using otolith microchemistry in ecological investigations. In particular, I highlight aspects of otolith microchemistry concerning the biological nature of otoliths (i.e., rate of elemental deposition, crystal structure, and elemental deposition with regards to life history stage) as well as the technical nature of their analysis (i.e., use of certified reference material and the three dimensional structure of otoliths) to improve the accuracy and precision of the assay.

Co authorship

Several people have been important in the development of this thesis and as a result have been included as co-authors for some of the chapters. It is therefore necessary for me to highlight each author's contributions to these chapters. In all of these chapters, I was the sole developer of ideas, data analysi, and writer, while aspects of data collection (both field and lab) were shared with others. I acknowledge their specific contributions below:

Peter Sale: Peter has been important in the development of ideas found throughout this thesis, but was particularly helpful in the design of experiments discussed in Chapter 4.

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Over the years, my colleagues have been the source of inspiration that helped develop various aspects of this thesis. In recognition of their assistance, I have made them co-authors on various chapters.

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Paolo Usseglio: Paolo has been my field comrade for extended portions of the 2002 and 2003 summers, and thus has provided invaluable field assistance particularly relating to Chapters 2, 4, and 6. In addition, he has provided editorial assistance with Chapter 6.

James Derek Hogan: Derek, another field comrade, helped with field collections used in Chapters 2, 3 and 4. He has also helped in the editorial process of Chapter 4.

Dedication

I dedicate this dissertation to Karen,
my best friend, wife, and healer,
and to Zeus,
my other best friend.

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Statement of originality

I declare that all the content in this thesis is original, and that all the research related to it is a product of my own imagination, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practises of the discipline.

Table of Contents

Abstract iii
Co authorship vii
Dedication ix
Acknowledgements x
Statement of originality xii
List of Tables xv
List of Figures xvii

Chapter 1: General introduction 1
Preamble 2
Life cycle 3
Thesis overview 7
Pelagic stage connectivity 7
Demersal stage connectivity 10
Thesis objectives 15
Study locations and organisms 20
Thesis structure 23
Bibliography 26

Section A: Pelagic stage connectivity 39

Chapter 2: Discrimination of *Stegastes partitus* populations and an assessment of the movement of individuals at Turneffe Atoll, Belize. 40
Abstract 41
Introduction 43
Materials and methods 46
Results 51
Spatial analysis 52
Temporal analysis 53
Connectivity analysis 54
Discussion 57
Acknowledgments 63
Bibliography 64

Chapter 3: Sagitta and lapillus: different microchemical recorders within the same fish. 79
Abstract 80
Introduction 81
Materials and methods 83
Results and Discussion 86
Acknowledgements: 91
Bibliography 92

Chapter 4: Ontogenetic variability in the otolith microchemistry of *Stegastes partitus*. 101
Abstract 102
Materials and methods 106
Results 112
Discussion 114
Acknowledgements 122
Bibliography 123

Section B: Demersal stage connectivity 131

Chapter 5: Discrimination of *Haemulon flavolineatum* from mangrove and coral reef habitats and an assessment of connectivity 132
Abstract 133
Introduction 134
Materials and methods 137
Results 142
Discussion 145
Acknowledgements 149
Bibliography 150

Chapter 6: Using otolith microchemistry of *Haemulon flavolineatum* (French grunt) to characterize mangroves and coral reefs throughout Turneffe Atoll, Belize: difficulties at small spatial scales. 159
Abstract 160
Introduction 161
Materials and methods 163
Results 167
Discussion 169
Acknowledgements 174
Bibliography 175

Chapter 7: General Discussion 183
Introduction 184
Biological processes that influence otolith microchemistry 189
Time to equilibrium 189
Development-related variability in otolith microchemistry 192
Crystal polymporphism 194
Embryonic development 194
Instrument-related factors 197
Reference materials 197
Otoliths as three-dimensional structures 199
Conclusion 200
Bibliography 202

Vita auctoris 208

List of Tables

Table 1.1: Simplified periodic table to illustrate changes in number of protons (i.e. atomic number, superscript) and number of protons and neutrons (i.e. atomic mass, subscript). Dotted lines denote missing cells. Note that copper (Cu) and zinc (Zn) have alternative isotopes with atomic masses 63 and 65 (for Cu) and 64 and 66 (for Zn)..... 36

Table 2.1: Dates of sampling periods in which newly settled *S. partitus* were collected from sites at Turneffe Atoll, Belize..... 70

Table 2.2: Elements included in statistical analyses that were determined from the coefficient of variation (CV) of NIST samples being < 10% and > 50% of otolith samples with values greater than detection limit. Average and standard deviation (SD) provided for detection limit by sample ablation, overall concentration, and concentrations per year. Average CV is of 46 different sets of otolith ablations (20 and 26 for 2002 and 2003, respectively). All values are in parts per million..... 71

Table 2.3: Univariate results of the nested MANCOVA, whereby site was nested within sampling period (SP) and year, while standard length (STDL) was the covariate... 72

Table 2.4: Results of the discriminant function analyses examining spatial and temporal variability in otolith edge microchemistry..... 73

Table 3.1: Elements included in statistical analyses that were determined from the coefficient of variation (CV) of NIST samples being < 10% and > 50% of otolith samples with greater than detection limit. Average and standard deviation (SD) provided for detection limit by sample ablation, overall concentration, and concentrations per year and otolith type. Average CV is of 25 different sets of otolith ablations. All values are in parts per million..... 96

Table 3.2: Results of the discriminant function analyses (DFA) to differentiate groups (i.e., locations/times of fish collections) using elemental concentrations of sagitta, lapillus, and sagitta plus lapillus. Specifically, information is provided regarding Wilks' Lambda (the variance not explained by the discriminant functions), Chi-square (X^2) statistic (the measure of statistical significance) and the proportion of the variance represented by significant roots (%). Factor correlations >|0.3| are indicated for each element (Li, Mg, Mn, Zn, Rb, Sr, and Pb) and root. 'S' and 'L' denote concentrations from sagittal and lapillar otoliths, respectively..... 97

Table 4.1: Average concentration (standard deviation, SD) of elements per sample (embryo otolith, juvenile otolith or water) per location/time. Ratios of concentrations of embryo otoliths: water samples and embryo otoliths:juvenile otoliths are provided along with average detection limit for embryo and juvenile otoliths, average coefficient of variation (CV) per element and percentage of samples greater than detection limit. Average CV is based on 25 different sets of otolith ablations..... 127

Table 4.2: Univariate results of the MANOVA comparing elemental concentrations among locations/times for a) embryos and b) juveniles. In addition, results are provided of a nested MANOVA, assessing c) ontogenetic variability in elemental concentrations between embryos and juveniles, which are nested within

locations/times of collection (*). Only elements with significant differences are included. 128

Table 5.1: Isotopes included in the DFA for Bahamas and Belize determined from coefficient of variation (CV) of NIST samples being < 10% and > 50% of otolith samples greater than three times detection limit. Average and standard deviation provided for detection limit by sample ablation and concentration (ppm). Average CV is the mean of CV of 5 (Belize 2002) or 6 (Bahamas 2001 and Belize 2001) different sets of otolith ablations. All values in parts per million. 154

Table 5.2: Classification matrix for the spatial analysis of each of Bahamas 2001, Belize 2001 and Belize 2002, from their respective DFA. Also included are the DFA results of the assessment of temporal variability (i.e., temporal analysis) in otolith microchemistry as well as the estimation of fish movement between mangrove and reef sites (i.e., connectivity analysis). The temporal variability in microchemistry attempted to classify fish collected in 2002 based on the DFA produced from Belize reefs in 2001 data. For the connectivity analysis, the otolith chemistry for the juvenile portion of fish collected from reefs in Belize 2002 were compared to the otolith edge chemistry of fish from the three sites in 2001. 155

Table 5.2: Classification matrix for the spatial analysis of each of Bahamas 2001, Belize 2001 and Belize 2002, from their respective DFA. Also included are the DFA results of the assessment of temporal variability (i.e., temporal analysis) in otolith microchemistry as well as the estimation of fish movement between mangrove and reef sites (i.e., connectivity analysis). The temporal variability in microchemistry attempted to classify fish collected in 2002 based on the DFA produced from Belize reefs in 2001 data. For the connectivity analysis, the otolith chemistry for the juvenile portion of fish collected from reefs in Belize 2002 were compared to the otolith edge chemistry of fish from the three sites in 2001. 155

Table 6.1: Elements included in statistical analyses that were determined from the coefficient of variation (CV) of NIST samples being < 10%, and > 50% of otolith samples greater than detection limit. Average and standard deviation (SD) provided for detection limit by sample ablation and overall concentration (parts per million). Average CV is the mean of CV of 15 different sets of otolith ablations. 179

List of Figures

Figure 1.1: Temporal trend of refereed biological publications pertaining to otolith microstructural and microchemical work. Literature searches were conducted using Cambridge Scientific Abstracts Internet Database Service. Two searches were performed: the first to determine the total number of otolith microchemical and microstructural papers per year using the following text = keywords (otolith*) and microstr* or microchem*) or title otolith*; the second to determine the total number of otolith microchemical papers per year using the following text = keywords (otolith*) and keyword (microchem* or element*) or title (otolith*) and keyword (microchem* or element*). Plot of otolith microstructural papers was determined from the subtraction of both searches. Influential discoveries are noted..... 37

Figure 1.2: Maps of study regions (Belize, Mexico, and Bahamas) and locations (Turneffe Atoll, Banco Chinchorro, and Lee Stocking Island, respectively). Ovals represent location of regions..... 38

Figure 2.1: Map of Turneffe Atoll, Belize, showing sites (filled circles) from which *S. parritus* were collected. Crosses indicate locations of development (in the form of resorts and one field station)..... 74

Figure 2.2a: Adjusted elemental concentrations (ppm) of otolith edges per site for each sampling period, and year. Means and 95% confidence limits are presented. 75

Figure 2.2b: Adjusted elemental concentrations (ppm) per site of otolith edges for each sampling period, and year. Means and 95% confidence limits are presented. 76

Figure 2.3: Patterns of larval dispersal among sites at Turneffe Atoll in 2002 sampling periods 1 (a), 3 (b) and 5 (c), and 2003 in sampling periods 1 (d), 3 (e), and 5 (f). A solid line indicates an individual's movement away from the site it was spawned to the site it was collected. A dashed line indicates that an individual settled where it was spawned. Each line (dashed or solid) represents the movement of one individual. Only those individuals from DFA of test data set that had posterior probabilities >90% are indicated. n denotes the number of individuals whose natal origin was estimated. 77

Figure 2.4: Frequency and proportion histogram of the estimated dispersal distance of individuals. Only those individuals from DFA of test dataset that had a *posterior probability* >90% are illustrated. 78

Figure 3.1: Map of study locations (Turneffe Atoll, Belize and Banco Chinchorro, Mexico). Triangles and circles correspond to sampling that was conducted in 2002 and 2003, respectively. 98

Figure 3.2: Differences in concentrations (ppm) between lapillar and sagittal otoliths for each fish (each bar represents one individual), in terms of Li, Mg, Mn, Zn, Rb, Sr, and Pb. Results are provided for the Wilcoxon matched pairs tests, which assessed whether there were significant differences in elemental concentrations between otolith types. BC and TA refer to Banco Chinchorro, Mexico and Turneffe Atoll, Belize, respectively..... 99

Figure 3.3: Plot of individuals from their locations/times of collection in DFA space using elemental concentrations from both otolith types. Elements are indicated that account for > 10% of the separation. 100

Figure 4.1: Map of study locations, Turneffe Atoll, Belize and Banco Chinchorro, Mexico and sites of embryo and juvenile collections. 129
Figure 4.2: Plots of embryo (a) and juvenile (b) fish from their sampling locations/times in DFA space using elemental concentrations of their otoliths. Elements are indicated that account for > 10% of the separation. Inset is an image of the laser (left oval) approaching a ~14μm diameter embryo otolith (right circle). 130

Figure 5.1: Location of enclosure sites at Lee Stocking Island, Great Exuma, Bahamas (2 reef and 1 mangrove) and Calabash Caye, Turneffe Atoll, Belize (1 reef and 2 mangrove). 156
Figure 5.2: Trace element concentrations (ppm) showing differences among sites within a region and year as determined by ANCOVA. Different letters indicate significant differences in pairwise comparisons from Bonferri post hoc test. Analyses were performed using $\log_{10}$ transformed data, but unstandarized data are presented. 157
Figure 5.3: Plot of factor scores produced from the DFA of otolith chemistry of fish held at three sites in a) Bahamas 2001, b) Belize 2001, and c) Belize 2002. Elements that account for a substantial amount of variability in the discrimination are indicated. For Bahamas 2001, although function 2 did not significantly improve the discrimination it was included for illustrative purposes. 158

Figure 6.1: Geographic position of mangrove and coral reef sites throughout Turneffe Atoll, Belize. Triangles and circles indicate mangrove (MG) and coral reef (RF) sites, respectively. Site sample sizes provided in adjacent table. 180
Figure 6.2: Adjusted $\log_{10}$ transformed otolith elemental concentrations (ppm) of Li, Mg, Zn, and Rb per habitat and site. Mean and standard error are plotted. Statistical significance from nested MANCOVA is indicated. 181
Figure 6.3: Spatial gradient of otolith elemental concentrations (ppm) of Li, Mg, Zn, and Rb throughout Turneffe Atoll, Belize. Contour plots are of untransformed concentrations (ppm). Triangles and circles indicate mangrove (MG) and coral reef (RF) sites, respectively. 182

Chapter 1: General introduction

Preamble

Ecology is the scientific study of the interactions that determine the distribution and abundance of organisms (Krebs 1994), and since organisms are not eternal, extinction is expected unless populations are replenished by the addition of new organisms (Sale 2002). The maintenance of any population of a species can either be through the contribution of offspring produced within it or by the delivery of individuals from other populations. Of interest to ecologists are the factors and processes related to the delivery of propagules that influence the distribution and abundance of individuals.

Fishes that occupy coral reefs form the richest vertebrate communities on the planet (Choat and Bellwood 1991), yet because of increasing exploitation from industries such as commercial and sport fishing, tourism, and the aquarium trade, as well as indirect effects from coastal development and degradation, these systems are being threatened. Therefore, a comprehensive understanding of the processes involved in structuring reef fish populations is crucial to facilitate effective management. Historically, reef fish ecologists focused on processes, such as competition and predation, operating during the juvenile and adult stages (post-settlement stage), which were suspected to be critical in determining the distribution and abundance of individuals (Jones 1991). However, in the mid-70's, theories of what structured reef fish populations shifted from the post-settlement view to one that included all aspects of the life-history (i.e., pre- and post-settlement stages; settlement is defined as the movement of an individual from pelagic to demersal habitat as is seen in fish with a bipartite life cycle, Mora and Sale 2002). This change was the result of growing awareness of the relevance of larval (pre-settlement stage) survival and dispersal in determining the distribution and abundance of organisms (Sale 1991).

In the 70s, Sale proposed the lottery hypothesis in which, for the first time the patterns of occurrence of apparently competing species of reef fish resulted from variability in the recruitment (recruitment, defined as the addition of a cohort to a population. Mora and Sale 2002) of larvae and not from variability in adult abundance and fecundity (Sale 1977, 1978). The lottery hypothesis suggested that larval abundance was sufficient to fill available habitat space, but when space was made available (through the movement or death of an adult), a randomly (hence the term "lottery") selected individual drawn from the pool of available species would become resident. In the early 80's a derivative of the lottery hypothesis was proposed, referred to as the recruitment limitation hypothesis (Doherty 1981). The recruitment limitation hypothesis suggested that larval abundance was not sufficient to saturate available space on the reef (lottery hypothesis), but instead their abundance was well below limits set by resource availability (Doherty 1981; Richards and Lindeman 1987; Doherty and Williams 1988; Robertson et al. 1988). Consequently, the distribution and abundance of adults in a habitat was determined by spatial and temporal variability in the number of settling larvae and adult populations did not achieve or exceed resource availability. Because of these ideas, the ecology of reef fish (i.e., the study of their distribution and abundance) was best understood through knowledge of the entire life cycle, including the dispersive larval phase.

Life cycle

The vast majority of coral reef teleosts have a life cycle in which a pelagic larval stage is the first of two distinct phases. Because the pelagic larval stage can last from 9 to 100 days depending on the species (Leis 1991 and references therein), larvae may

disperse up to 100s of kilometres, and thus likely determine the geographical extent of adult populations (Leis 1991). Yet, the number of larvae present is the result of several factors operating synergistically (see Cowen 2002 for more detail). For instance, the number of larvae that begin their pelagic stage as well as when and where this occurs, is influenced by the spatial distribution and abundance of spawning adults, their fecundity (e.g., approximately 3000 eggs/nest for *Pomacentrus amboinensis*, McCormick 2003) and the timing of spawning. Next, oceanographic circulation patterns (Sale 1980; Victor 1984; Richards and Lindeman 1987; Cowen and Castro 1996; Limouzy-Paris et al. 1997), temperature (Shenkar et al. 1993), food availability (for larvae, Richards and Lindeman 1987; and for breeding females, McCormick 2003), and predation pressure (Richards and Lindeman 1987; Cole and Sadovy 1995) further influence the number of surviving larvae and their spatial and temporal distribution. Ontogenetic changes in larval behaviour can result in very different dispersal possibilities and thereby further modify the spatial and temporal abundance of larvae. For instance, early stage pelagic larvae are more or less passive particles while late stage larvae are effective swimmers capable of sustained and directed swimming (Leis 1991; Stobutzki and Bellwood 1994; Leis et al. 1996; Stobutzki and Bellwood 1997; Leis and Carson-Ewart 1998; Stobutzki 1998; Leis and Stobutzki 1999; Fisher et al. 2000; Fisher and Bellwood 2002; Leis 2002; Leis and Carson-Ewart 2003).

Following the pelagic period, surviving larvae will undergo morphological, behavioural and physiological changes that promote their settlement to a habitat such as a coral reef, seagrass bed, sand or mangrove, where they will commence their juvenile existence (demersal stage) and remain for most if not all of their remaining life. The

abundance of demersal individuals is then further restricted by predation (Hixon and Carr 1997; Steele 1998; Holbrook and Schmitt 2003) and competition (Steele 1998). For instance, Holbrook and Schmitt (2002), using an infrared video system, observed the loss of damselfish during their first hours on the reef due to predation. Although competition rarely kills fish directly, it still can play an important role influencing abundance of individuals. For example, Kami and Ikehara (1976) observed that the schooling behaviour of an unusually large settlement of rabbitfish overgrazed areas that subsequently led to mass starvation of the population.

Clearly, factors influencing both pelagic and demersal stages determine the distribution and abundance of reef fish at any given time. However, what maintains a reef fish population through time? The continuance of a population is made possible by the replenishment of individuals such that mortality and emigration are balanced or exceeded. Population replenishment may occur through the influx of individuals produced locally (i.e., self-recruitment), in which case the population is considered closed (majority of new individuals added to a population were spawned by that population, Mora and Sale 2002). Alternatively, replenishment may occur through the influx of larvae originating from neighbouring populations (i.e., dispersal) in which case the population is considered open (majority of individuals added to a population were spawned elsewhere, Mora and Sale 2002). The latter type of replenishment provides for population connectivity, which is defined as the demographic link maintained between neighbouring populations of a species due to dispersal of individuals between them (Mora and Sale 2002). Clearly, closed and open populations are at the extremes of a continuum in which any population is likely maintained by the product of propagules

originating both near and far. Knowledge of the patterns of replenishment are essential for a basic understanding of the population dynamics, while the scale at which populations have a significant interconnectivity is critical for the design and management of marine protected areas (Williams et al. 1984; Doherty 2002). Given the pressures on coral reef systems and associated shallow habitats and the necessity for more efficient management, there is growing need to understand the scales at which populations are connected, and the proportion of individuals transferred between populations.

Over the last 10 years, a shift has occurred in the way reef fish ecologists have perceived population replenishment relative to both pelagic and demersal stages. Specifically, as a result of novel work by Swearer et al. (1999) and Jones et al. (1999) (discussed in more detail below) the classic view of reef fish populations as open, highly connected networks of populations maintained by pelagic stage long distance dispersal, has been replaced by one that suggests the potential for closed populations due to the occurrence of larval retention to their natal reef. With respect to demersal stage reef fish ecology, the almost universal acceptance that adult populations of certain species are maintained through connectivity with nearby shallow water habitats, such as mangroves (often referred to as nursery habitat; habitats believed to be important for the survival and development of juveniles that later supplement populations elsewhere) has come under scrutiny. In particular, recent studies have argued that there are many exceptions to the nursery role concept (see Edgar and Shaw 1995; Heck et al. 1995). According to reviews by Beck et al. (2001), Gillanders et al. (2003), and Sheridan and Hays (2003) (discussed in more detail below), the limited amount of direct evidence for the nursery role of

mangroves and seagrass beds prohibits any declaration of their universal relevance to the maintenance and sustainability of reef fish populations.

Thesis overview

The purpose of this thesis was to use otolith microchemistry to address issues of connectivity relating to both pelagic and demersal stages of the reef fish life cycle. Specifically, in terms of pelagic stage connectivity, I investigated the spatial scales at which reef fish populations were connected by larval dispersal, using recently hatched and settled *Stegastes partitus* (Bicolor damselfish). Concerning demersal stage connectivity, I addressed whether juvenile *Haemulon flavolineatum* (French grunts) use mangrove habitats as nurseries from which they later move to occupy nearby coral reefs.

Pelagic stage connectivity

Traditionally, reef fish larvae were regarded as passive particles. The spatial extent of travel by these larvae was largely determined by the combination of ocean currents and time spent as pelagic larvae (referred to as pelagic larval duration), whose relatively small body size was assumed to translate into a lack of directional control of their trajectory (Leis 2002). As a result, populations were viewed as being open, that is, they received larvae produced by spawning at distant locations (Sale 1980; Williams et al. 1984; Doherty and Williams 1988; Sale 1991). However, recent novel experimental and modelling studies have questioned the openness of reef fish populations by suggesting that populations receive propagules spawned locally. That is, individuals hatched from a population in a given area remain nearby or return to the population at the completion of larval life. For example, using differences in growth rate and trace-

element concentrations in otoliths, Swearer et al. (1999) estimated that 44.5% of the recruitment of *Thalassoma bifasciatum* (Bluehead wrasse) larvae to St. Croix, U.S.V.I., had developed within coastal waters and thus were the result of local self-recruitment (i.e., they exist as substantially closed populations). In a study by Jones et al. (1999), approximately 10 million embryos of *Pomacentrus amboinensis* (Ambon damselfish) were tagged using tetracycline at a number of sites around Lizard Island, Great Barrier Reef, Australia. Tetracycline becomes incorporated into hard structures including otoliths, and fluoresces under ultraviolet light. Of 5000 captured larvae, 15 were found to contain the tetracycline tag and based on the number of spawning adults and their fecundity, 15-60% of recruits were estimated to have been produced locally (i.e., 40-85% produced elsewhere).

Cowen et al. (2000) also highlighted the importance of self-recruitment to reef fish populations using a 2D hydrodynamic model of Barbados together with estimates of larval mortality and passive diffusion (see Boehlert et al. 1992 and Cowen and Castro 1994 for evidence of retention). Specifically, Cowen et al. (2000) observed that when their model included realistic advection and diffusion components, larvae were transported over a large geographic area (approximately 10^6 km^2), suggesting the prevalence of open populations for a large number of species that have a pelagic larval phase in common. However, when the same model included larval mortality the resulting scenario showed little or no surviving larvae transported away from their origin and thus a lack of population openness was observed. Although the conclusions of Cowen et al. (2000) were insightful, the model was criticized for its oversimplification of passive larvae dispersal (see Warner et al. 2000) since there is considerable evidence to

suggest that larvae are able to augment their dispersal through behaviourally modified swimming (see Stobutzki and Bellwood 1997; Leis and Carson-Ewart 1998; Stobutzki 1998).

According to Leis (2002), the assumption of passive dispersal is appropriate only immediately following hatching when larvae are their smallest and least developed, but this is also when larvae could be retained because of hydrodynamic processes such as eddies. For instance, in both 2D and 3D hydrodynamic simulation models produced by Black and coworkers (Black and Moran 1991; Black et al. 1990, 1991; Black 1993), a large proportion of passive particles released over a reef were observed to be retained for several days due to local hydrodynamics. Swearer et al. (2002) drew on several lines of evidence, including dispersal of invasive species and geographic patterns in population genetics, to suggest that there were strong reasons to re-evaluate the open population paradigm. For instance, using introduced fishes to the Hawaiian Islands and a species of barnacle spreading throughout Europe as examples, Swearer et al. (2002) argued that the establishment of these populations must be a consequence of self-recruitment since no other source populations were present to assist in colonization. In addition, Swearer et al. (2002) identified several studies that used population genetics to observe distinct population structures over relatively small spatial scales (see Taylor and Hellberg 2003 for an investigation of *Elacatinus evelynae*, cleaner goby), again suggesting the prevalence of self-recruitment.

Since only a few studies, at limited locations (i.e., those highlighted here: St. Croix, Lizard Island, and Barbados), have provided evidence for the importance of self-recruitment in maintaining reef fish populations, the degree to which retention is

important to population replenishment will not be clear until other regions (with a greater number of up-current reef systems) are investigated (Swearer et al. 2002). Mora and Sale (2002) cautioned against impulsive conclusions that reef fish populations are solely maintained by larval self-recruitment since the limited evidence suggests a combination of retention and dispersal (15-60% retention versus 40-85% dispersal in the study by Jones et al., 1999). They further note that whether a population behaves as open or closed will likely depend upon its location (and therefore specific hydrography), species (with specific larval duration, behaviour and capabilities) and the spatial and temporal scale at which its behaviour is examined (Mora and Sale 2002).

Demersal stage connectivity

Following an individual's transition from the pelagic environment to a demersal existence, differences in mortality likely occur depending on the shallow water habitat selected. This variation in mortality among habitats will often result from differences in the quality and/or quantity of resources, abundance of predators and/or frequency of visits, or a host of other biological and environmental factors. That is, the magnitude of the impact post-settlement processes have on a fish's survival will likely vary depending on the habitat that an individual settles. For instance, the influence of predation was apparent when Shulman (1985) showed that mortality of juvenile grunts declined with greater distance from small patch reefs harbouring predators. In addition, Wellington (1992) and Holbrook and Schmitt (2003) observed that survival of newly settled reef fish varied depending on the habitat they selected (fore reef versus back reef or reefs that varied in their structural attributes, respectively). These examples, as well as many others (see below) have suggested that microhabitat features (i.e., food availability, complexity,

shade etc.) can influence the survival of individuals and the maintenance of their numbers (Hixon and Beets 1989, and 1993). Consequently, certain habitats (referred to as nursery habitats) may increase the probability of an individual's survival through the provision of food and/or shelter, and thus increase the likelihood of that individual contributing to nearby adult populations.

Mangroves and seagrass beds are shallow water habitats that often have greater abundance of juvenile reef fish relative to populations on coral reefs. Often, these habitats are referred to as nurseries because of the apparent increase in juvenile survival that these abundance patterns suggest (see Robertson & Duke 1987; Dennis 1992; Haliday & Young 1996; Gillanders 1997a and b; Nagelkerken et al. 2000a,b, 2002; Nagelkerken and van der Velde 2002). The greater abundance of juveniles within these habitats has been attributed to several features that could enhance survivorship. For instance, habitats may offer greater abundance of food and food types for developing organisms (Odum & Heald 1972; Stunz et al. 2002; Cocheret de la Moriniere et al. 2003). Complexity, shade and/or turbidity-reduced visibility inherent to the mangrove and seagrass habitats may reduce the efficiency of predation (complexity - Quinn & Kojis 1985; Parrish 1989; Cocheret de la Moriniere et al. 2002; Adams et al. 2004; shade - Cocheret de la Moriniere et al. 2002; visibility/turbidity - Blaber & Blaber 1980). Finally, predators may frequent mangrove and seagrass habitats less than coral reefs (Sasekumar et al. 1984; Shulman 1985; Parrish 1989). However, inherent to the nursery habitat concept is not only that nursery areas should have higher juvenile densities, reduced predation, and abundant resources, but that juveniles develop and later move to nearby adult habitats (e.g., coral reefs) to supplement these populations (Beck et al.

2001). Unfortunately, such movements have rarely been included in the working definition of a nursery (but see Dennis 1992; Beck et al. 2001; Gillanders et al. 2003). If a habitat functions as a nursery there must be quantification of a) movement of individuals between juvenile and adult habitats, b) density of juveniles per unit area must be greater in the nursery habitat, and there must also be observed c) growth and d) survival advantages for juveniles within the nursery habitat (Beck et al. 2001).

To assess the movement of individuals between juvenile and adult habitats researchers take advantage of technologies such as data loggers, stable isotopes, genetic markers and otolith microchemistry (Beck et al. 2001). Using stable isotopes, Fry (1981) identified seasonal movements of *Penaeus aztecus* (Texas brown shrimp), which suggested that shallow water habitats supply more shrimp to south Texas offshore fisheries than do deeper estuarine bays. Similarly, Fry et al. (1999) noted the importance of seagrass and mangroves-lined bays since approximately 40% of *Farfantepenaeus duorarum* (Pink shrimp) collected offshore were classified as recent immigrants that likely originated from seagrass beds or mangrove-lined bays. Thorrold et al. (1998) used elemental concentrations of trace elements and stable isotopes to classify juvenile *Cynoscion regalis*, (Weakfish), to their natal estuary with a 60-70% success rate. Later, by examining adults spawned during the initial collection, they identified that 60-81% of the analysed individuals showed signs of site fidelity (i.e., adults returned to the estuary in which they were spawned) (Thorrold et al. 2001). Gillanders and Kingsford (1996) and Yamashita et al. (2000) suggested anywhere from 32-65% and up to 89% (Gillanders 2002) of the fish sampled on rocky reefs had occupied a nursery habitat (i.e., estuarine seagrass bed in Gillanders and Kingsford (1996) and Gillanders (2002), and estuary and

inshore nursery in Yamashita et al. (2000)) as juveniles. However, estimates of connectivity were likely overestimated since these studies limited the origination of individuals to one of two to four sites (see Gillanders and Kingsford 1996; Yamashita et al. 2000; Gillanders 2002).

Using reef fish biomass and fishing pressure, Mumby et al. (2004) indirectly quantified the importance of mangroves to Caribbean reef fish. Specifically, Mumby et al. (2004) showed that the biomass of each of *Haemulon sciurus* (Bluestriped grunt), *Ocyurus chrysurus* (Yellowtail snapper), *Scarus iseri* (Striped parrotfish), *Lutjanus apodus* (Schoolmaster) and *H. flavolineatum* (French grunt) was significantly greater on patch reefs in mangrove-rich areas than mangrove-scarce areas. Furthermore, using *S. guacamaia* (Rainbow parrotfish), a species predominantly associated with mangroves, they observed few individuals on reefs with limited nearby mangroves, suggesting that mangroves are important to the abundance of individuals of this species. Similarly, Nagelkerken et al. (2002) noted that 11 of 17 species previously reported to use mangroves and seagrass beds as nurseries (see Negelkerken et al. 2000a,b and 2001), were absent or at lower densities on reefs of islands lacking mangroves and seagrass beds, again implying movement between habitats (also see Dorenbosch et al. 2004). However, Halpern (2004) indicated that size of mangrove stands and proximity to adult reef habitat did not affect adult fish densities of *Gerres cinereus* (Yellowfin mojarra) and *L. apodus* on coral reef patches.

Comparisons of growth and survival advantages for juveniles occupying seagrass beds or mangroves and coral reefs habitats have sometimes provided support for the labelling of certain habitats as nurseries. For instance, in a recent review of seagrass beds

as nursery habitats, Heck et al. (2003) noted that approximately half of the studies (of 11) showed juveniles (3 fish and 2 invertebrate species) exhibited greater growth rates in seagrass beds than in other habitats (other habitats defined by Heck et al. as unvegetated structure, no structure, and vegetated structure). Acosta and Butler (1997) investigated predation on newly settled *Panulirus argus* (Spiny lobster), along the Belize Barrier Reef and observed greater predation in seagrass and coral crevices than among mangrove prop roots. However, because larger juveniles had lower predation in mangroves and coral patches than in seagrass, the survival of larger juveniles was suggested to depend on their proximity to fringing mangroves and adjacent coral crevice shelters. Based on fish tether experiments at Lee Stocking Island, Bahamas, Dahlgren & Eggleston (2000) indicated that *Epinephelus striatus* (Nassau grouper) juveniles occupy algal beds because they experience lower levels of mortality. Kramer (1991), working on *Paralichthys californicus* (California halibut) in shallow coastal and bay habitats of California, reported that juveniles had higher survival during the first year of life if they settled along the coast and entered and used the bays as nursery areas.

By contrast, several studies have argued that seagrass or mangrove habitats do not necessarily function as nursery habitat. For example, Blaber & Milton (1990), working in Solomon Islands, and Morton (1990), in Eastern Australia, noted little reduction in predation pressure between an estuary and coral reefs and between mangroves and adjacent habitats, respectively, since top-level piscivores were present in both habitats. In addition, work in Senegal by Vidy (2000), showed a lack of support of mangroves as nurseries since there was relatively high predation pressure by *Hemichromis fasciatus* (Banded jewelfish) on young fish, shrimps, and crabs in mangroves. Finally, Chittaro et

al. (2005) suggested that generalization about the nursery potential of mangrove and seagrass beds should be avoided since their investigation revealed substantial variability among replicates of mangrove, seagrass and coral reef habitats, in Belize and Mexico, in terms of juvenile density, assemblage similarity, and relative rates of predation of juvenile fish. In a recent review, Gillanders et al. (2003) noted that speculation about nursery potential should be avoided because of the limited direct evidence detailing the movement of fish between suspected nursery and adult habitats. The relevance of these habitats to the long-term maintenance and sustainability of these reef populations remains unclear and therefore more detailed investigations are necessary to understand whether certain habitats, such as mangroves and seagrass beds, operate as nurseries for reef fish.

Thesis objectives

Our uncertainty about the levels of both pelagic and demersal stage connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals; Mora and Sale 2002) highlights a significant gap in our knowledge of what determines the distribution and abundance of coral reef fish. This is a serious gap, given the degree of anthropogenic pressure on fish stocks (i.e., overfishing) and the current rate of environmental degradation worldwide (Ronnback 1999). If management and conservation efforts are to have any significant positive impacts on the ecologically and economically important resources of reef fish, then it is essential for us to understand the spatial and temporal scales at which populations are structured and the degree of connectivity among them (Mora and Sale 2002). The goal of this thesis was to assess the degree of connectivity among populations of two highly abundant Caribbean reef fish (*Stegastes partitus*, Bicolor damselfish and *Haemulon flavolineatum*, French

grunt) at specific, but different portions of their life history; pelagic and demersal stages. To conduct this investigation experiments used microchemistry of fish otoliths.

Otoliths are paired structures present as three types (sagitta, lapillus, and astericus; listed in decreasing size), located within fluid (endolymph) filled sacs of the inner ear system, and are found within most teleosts. Otoliths are primarily used for sound reception and orientation in a three dimensional environment (Campana 1999; Sollner et al. 2003; Lombarte and Popper 2004), and their formation has been identified for several species to occur prior to hatching (this portion of the otolith is referred to throughout this thesis as the otolith core). For instance, Pisam et al. (2002), studying *Danio rerio* (Zebrafish), noted otolith formation 30 hours following fertilization (hatching for this species is 2 days after fertilization), while Zhang and Runham (1992) observed otolith formation on day 3 (hatching is 5 days after fertilization) in *Oreochroms niloticus* (Nile tilapia).

Prior to the 1960's (and as early as 1800's; see Jones 2002), biologists using the unique properties of otoliths (i.e., their yearly banding pattern) determined the age of fish and hence the age structure of populations (Yoshihara 1955; Dannevig 1957; Hotta 1960). It was not until the 1970's, when Panella (1971) observed daily increments in otoliths, that otolith studies became prevalent in fisheries research (Figure 1.1). As a result of the discovery of daily increments it was possible to use the microstructure of otoliths to assess patterns of population age structure and estimate mortality and growth rates particularly for juvenile fish.

Structurally, the banding pattern of otoliths that produces the daily increments results from alternating layers of calcium carbonate ($CaCO_3$) and protein that are

deposited throughout the life of the individual. Unlike scales and vertebrae, which have been used to age fish, otoliths are metabolically inert and therefore are ideal for retrospective analyses (Campana 1999, Campana and Thorrold 2001). Because of the inert properties of otoliths, scientists in the late 70's became interested in their chemical nature (Macpherson and Manriquez 1977; Papadopoulou and Moraitopoulou-Kassimati 1977; Papadopoulou et al. 1980) (Figure 1.1). As technology for chemical analyses evolved scientists determined that as alternating layers of calcium carbonate and protein were deposited, trace elements (e.g. Sr, Ba, Mg, Mn etc.) were incorporated. The incorporation of elements into an otolith resulted by way of substitution for Ca, inclusion within the growing crystal, or attachment to the organic matrix (Campana 1999; de Pontual and Geffen 2002).

The substitution of any element for calcium is determined by their similarity in terms of atomic size and charge, but is also influenced by an element's concentration relative to Ca (e.g., Sr is unlikely to inhibit Ca uptake and thus Sr incorporation is likely dependent upon its concentration in the surrounding water) (Kraus and Secor 2004). Because neutrality of charge is required for any molecule (in this case CO_3^{-2}), certain elements of the periodic table are more readily substituted for Ca than others (i.e., elements within Group 2 of Table 1.1 are similarly charged to Ca). However, each successive row (i.e., period) in the periodic table represents an element with a larger atomic mass (i.e., greater number of protons and neutrons), which may restrict it from substituting for Ca because of its larger ionic size (de Pontual and Geffen 2002).

The size of an element may also influence whether it can be trapped within the interstitial spaces of the growing otolith. As a result, elements such as Na, Cl, Zn and K

are not likely to be incorporated into interstitial space because their size is too large (de Pontual and Geffen 2002). However, Gauldie et al. (1998) reported the movement of relatively large ions from a dye (di-methyl arsenate) into interstitial spaces of otoliths. In addition to this lack of information regarding the deposition of elements into interstitial spaces, we understand less well the incorporation of trace elements in the organic matrix (Campana 1999; de Pontual and Geffen 2002).

Although some information is available about the mechanism of incorporation of trace elements from the endolymph onto the otolith surface very little is understood about the path prior to this (i.e., from the external environment to the internal environment of the fish). To address issues of initial uptake of elements, several studies have investigated whether trace elements found within otoliths are the product of dietary or environmental uptake (i.e., respiration and/or osmoregulation). Many of these studies have indicated that concentrations of several elements in otoliths reflect that present in the environment occupied by the fish (e.g., Sr- Farrell and Campana 1996; Gallahar and Kingsford 1996; Bath et al. 2000; Milton and Chenery 2001; Kennedy et al. 2002; Elsdon and Gillanders 2003; Ba- Bath et al. 2000; Milton and Chenery 2001; Elsdon and Gillanders 2003; Li- Milton and Chenery 2001; Pb- Geffen et al. 1998; and see Wells et al. 2000 and 2003 for evidence of trace element relationships between scales and water). However, what influences the incorporation of an element from the environment into an otolith?

As an ion of an element moves from the water into the blood plasma, endolymph, and finally onto the growing otolith, a series of filters can decouple the environmental concentrations from that of the otolith. For instance, elemental filtration likely begins at

the gills during respiration and/or at the intestine during osmoregulation (marine fish swallow water to regulate their salinity). Elements incorporated into the blood will encounter another filter, this time between the blood and endolymph, whereby the concentrations of all major ions are depleted because of physiological requirements (Kalish 1991; Campana 1999; Thorrold and Hare 2002). Specifically, elements such as P, Cu, S, and Ca are strongly regulated and therefore concentrations are uniform despite variability in environmental concentrations (Campana 1999). The final level of elemental filtration occurs between the endolymph and the growing surface of the otolith, where, as previously mentioned, elements are included if of a certain charge and/or size. Because filters are not suspected to noticeably impede the transfer of most trace elements (e.g. Mn, Fe, Zn, Sr, Ba, and Pb) (Campana 1999) scientists utilize their concentrations to identify the elemental signature of the environment that the fish occupied.

Because of the microstructural and microchemical nature of otoliths, the entire spatial history of a fish can be retrospectively determined. Consequently, otolith microchemistry has been used to directly quantify migratory pathways, the spatial arrangement of populations, and fish connectivity (e.g., Campana 1999; Campana et al. 2000; Milton and Chenery 2003; Hamer et al. 2003; Rooker et al. 2003). Because of this potential, otolith chemistry is a technique that is well suited for investigations concerning both pelagic and demersal stage connectivity in coral reef fishes.

The goals of this thesis were to investigate the use of otolith microchemistry to determine the degree to which reef fish populations are connected. The specific questions that were tested were whether reef fish populations of Tumeffe Atoll are maintained by larval recruits from nearby sites (i.e., pelagic stage connectivity) and

whether juveniles on coral reefs are supplied from nearby shallow water habitats such as mangroves (i.e., demersal stage connectivity).

Study locations and organisms

Work was conducted in the western Caribbean at Turneffe Atoll, Belize, and Banco Chinchorro, Mexico, and in the northeastern Caribbean at Lee Stocking Island, Bahamas (Figure 1.2). To investigate questions pertaining to pelagic stage connectivity, sampling was performed at fixed reef sites (each site encompassed a total area of approximately 0.040 km^2) within Turneffe Atoll and Banco Chinchorro. The studies associated with demersal stage connectivity were conducted at mangrove and reef sites (each of these sites encompassed a total area of approximately 200 m^2) at Turneffe Atoll and Lee Stocking Island. All locations are a substantial distance from a continental coastline (see below).

Turneffe Atoll is approximately 35 km from the coast of Belize, and is approximately 59 km long (north to south) and 18 km wide. A ring of coral reefs surrounds Turneffe Atoll that crest (i.e., near the surface), then gently descend to approximately 30 m depth, at which point there is a small plateau, after which it drops 100s of metres. The internal portion of Turneffe Atoll is composed of three lagoons, each of which contains a network of mangroves and seagrass flats that vary in depth from 0.25-5 m.

Banco Chinchorro is approximately 30 km from the Mexican coast and is 45 km long (north to south) and approximately 20 km wide (east to west). Similar to Turneffe Atoll, a ring of coral reefs surrounds Banco Chinchorro of a similar depth and slope. The

internal lagoon of Banco Chinchorro contains three islands (one centrally located and one at each of its northern and southern extremes).

Lee Stocking Island, Bahamas, is approximately 350 km from the nearest substantial land mass of Cuba and is one of several small islands that make up the Great Exumas. Lee Stocking Island is approximately 13 km long and 1km wide and sits between deep ocean water (>2 km depth) of Exuma Sound to its east and the shallow sands (1-5 m depth) of the Great Bahama Bank to its west.

At Turneffe Atoll, seven sites were permanently established in approximately 10 m of water and visited every other week (centred on new and full moons) starting in late May and ending in early September, in both 2002 and 2003 (Table 2.1, only those dates listed were used in analyses). At each visit to a site, 10-15 newly settled individuals (newly settled designated as fish less than 2.5 cm in total length) of *Stegastes partitus* were collected. In addition, embryos of *S. partitus* were collected from Turneffe Atoll and Banco Chinchorro in 2003. The spatial and temporal extent of these collections provides an opportunity to investigate variability in otolith microchemistry and patterns of connectivity at Turneffe Atoll.

Stegastes partitus (Class Actinopterygii, Order Perciformes, Family Pomacentridae) is a relatively small species (maximum size of approximately 10 cm) common on shallow coral reefs throughout the Caribbean. They are demersal spawners whose reproduction follows a lunar cycle (Knapp 1993). Approximately 3-5 days following a full moon females deposit a clutch of eggs (approximately 2000 eggs, Cole and Sadovy 1995) on the underside of coral, rock, or rubble in a male defended territory (Robertson et al. 1988; Knapp 1993). At sunset, 3.5 days after egg deposition, embryos

hatch and begin their 29 to 35 day pelagic existence (Robertson et al. 1988; Wellington and Victor 1989; Wilson and Meekan 2002). Following settlement, individuals are relatively sedentary, occupying a small territory for the remainder of their lives.

Stegastes partitus was chosen for this study because it is an abundant species that is relatively easy to catch with hand nets and clove oil. Furthermore, various aspects of their biology make them an ideal species to use in addressing issues of connectivity. For instance, because of their relatively sedentary adult existence (i.e., territorial behaviour Robertson et al. 1988) their otolith chemistry will document the elemental history for that area without any confounding effect that could be related to movement if larger, more mobile species were examined. In addition, since *S. partitus* readily occupy artificial substrate and are demersal spawners, it is possible to collect and manipulate embryos. This would not be the case for pelagic spawners.

To explore connectivity between mangrove and reef populations (i.e., demersal stage connectivity) sampling was conducted at Turneffe Atoll in 2001, 2002, and 2003 and at Lee Stocking Island, in 2001. Collections of French grunt, *Haemulon flavolineatum* (Class Actinopterygii, Order Perciformes, Family Haemulidae), a common Caribbean reef fish, were made throughout the two locations. French grunts are pelagic spawners that have a pelagic larval duration of approximately 15 days (Brother and McFarland 1981; McFarland et al. 1985) at the end of which they settle to various habitats including urchin spines, mangrove prop roots, seagrass beds and algal beds (Brothers and MacFarland 1981; Shulman 1985, 1987). Juveniles are commonly found in mangroves (Nagelkerken 2000a and b; Mumby et al. 2004) and are often observed to form schools that feed on invertebrates in the sand and seagrass beds at night. Adults, on

the other hand, are more solitary and are usually present on shallow reefs (Brothers and McFarland 1981; Nagelkerken 2000 a and b). Because of the commonly reported differences in habitat at different life stages and their widespread distribution and high abundance throughout the Caribbean, this species is ideal for investigations of the role mangroves play in maintaining reef populations.

Thesis structure

The main objective of this thesis was to provide insights into the discrimination of reef fish populations and the movement of individuals as they pertain to pelagic and demersal life history stages, by emphasizing experiments using trace elemental chemistry of otoliths. I have divided this thesis into two sections. First; section A examines issues relevant to pelagic stage connectivity of *S. partitus* within Turneffe Atoll, Belize. (fish from Banco Chinchorro were utilized in terms of population discrimination and investigations of ontogenetic variability), while section B investigates demersal stage connectivity of *H. flavolineatum* between mangrove and coral reef habitats at both Turneffe Atoll, Belize, and Lee Stocking Island, Bahamas.

Section A is concerned with whether otolith microchemistry can be used to a) identify populations, and b) establish a link between newly settled reef fish and the location from which they originated, but is also interested in c) what assumptions and refinements can be made to improve discrimination of populations and the understanding of fish movement. Specifically, in Chapter 2, otoliths of newly settled *S. partitus* were chemically analysed to assess the spatial and temporal scales at which otolith chemistry could discriminate populations and investigate connectivity among them. Using this information, connectivity was estimated by comparing the edge chemistry (which

corresponds to the elemental concentrations deposited prior to collection) from individuals collected throughout Tumeffe Atoll. to the core chemistry (which corresponds to the elemental concentrations deposited at the natal location) of individuals from a subsequent collection. In Chapter 3, both sagittal and lapillar otolith chemistries were explored as a means to improve the discriminatory ability of populations. Finally, in Chapter 4, I tested whether otolith microchemistry varied ontogenetically, and thus whether the use of core and edge chemistry to assess connectivity was an acceptable technique.

The second half of this thesis (Section B) was devoted to issues related to population discrimination and connectivity of individuals collected during their demersal stage. In particular, the focus was whether *H. flavolineatum*, collected from adjacent mangrove and reef habitats could be discriminated using otolith microchemistry. In addition, I questioned whether there was movement of fish between habitats to suggest that mangroves function as nurseries. To quantify any movement of fish from mangrove to reef habitats, Chapter 5 first tested whether there was significant variability in otolith microchemistry among fish held in mangroves and reefs, and then assessed the relative degree of connectivity between mangrove and reef habitats.

In Chapter 6, I assessed whether otolith microchemistry can discriminate wild *H. flavolineatum* collected from 19 sites throughout Tumeffe Atoll. Of particular interest was the degree to which fish from mangrove and coral reef sites could be discriminated.

Finally, in Chapter 7 (General discussion) I assimilate the ecological findings of both sections and discuss the application of otolith microchemistry as a tool for fisheries biologists and ecologists.

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Table 1.1: Simplified periodic table to illustrate changes in number of protons (i.e. atomic number, superscript) and number of protons and neutrons (i.e. atomic mass, subscript). Dotted lines denote missing cells. Note that copper (Cu) and zinc (Zn) have alternative isotopes with atomic masses 63 and 65 (for Cu) and 64 and 66 (for Zn).

Group	1	2	11	12	13	14	15	16	17	18
Period										
1	H_1^1									He_4^2
2	Li_7^3	Be_9^4								Ne_{20}^{10}
3	Na_{23}^{11}	Mg_{24}^{12}								Ar_{40}^{18}
4	K_{39}^{19}	Ca_{40}^{20}	Cu_{64}^{29}	Zn_{65}^{30}	Ga_{70}^{31}	Ge_{73}^{32}	As_{75}^{33}	Se_{79}^{34}	Br_{80}^{35}	Kr_{84}^{36}
5	Rb_{85}^{37}	Sr_{88}^{38}								
6	Cs_{133}^{55}	Ba_{137}^{56}								

Figure 1.1: Temporal trend of refereed biological publications pertaining to otolith microstructural and microchemical work. Literature searches were conducted using Cambridge Scientific Abstracts Internet Database Service. Two searches were performed: the first to determine the total number of otolith microchemical and microstructural papers per year using the following text = keywords (otolith*) and microstr* or microchem*) or title otolith*: the second to determine the total number of otolith microchemical papers per year using the following text = keywords (otolith*) and keyword (microchem* or element*) or title (otolith*) and keyword (microchem* or element*). Plot of otolith microstructural papers was determined from the subtraction of both searches. Influential discoveries are noted.

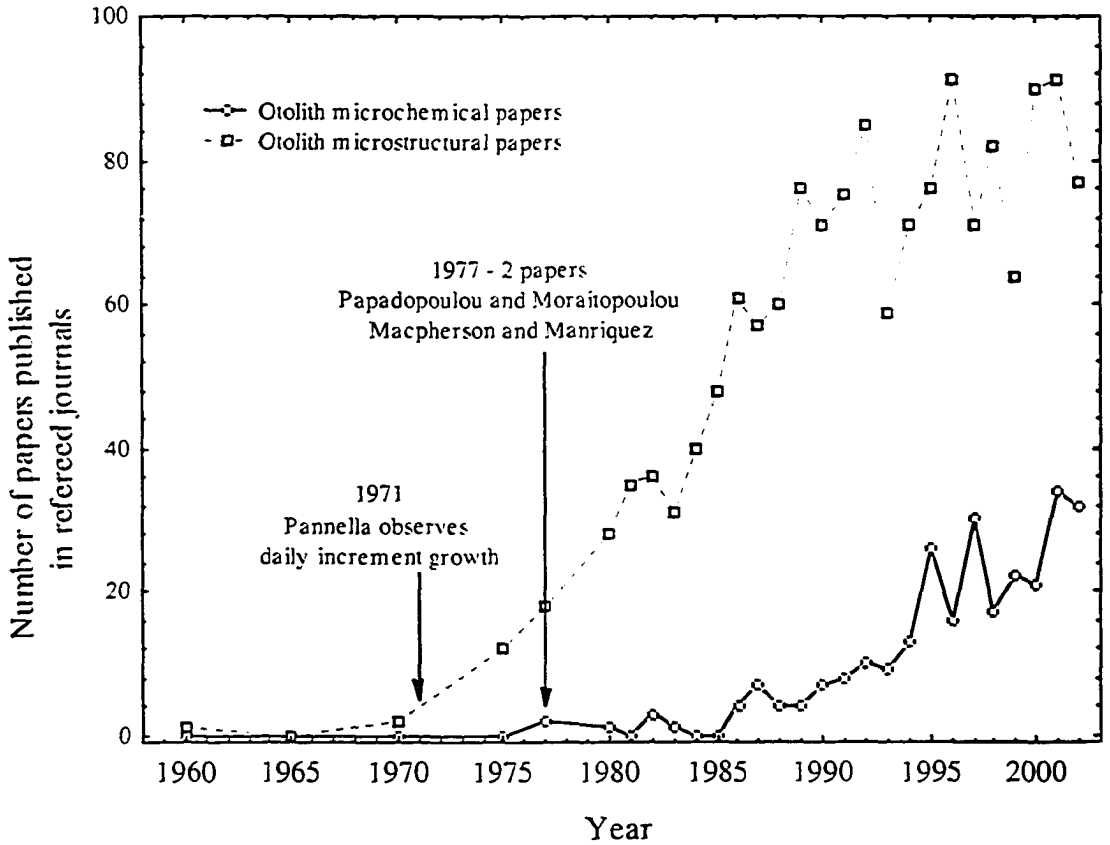
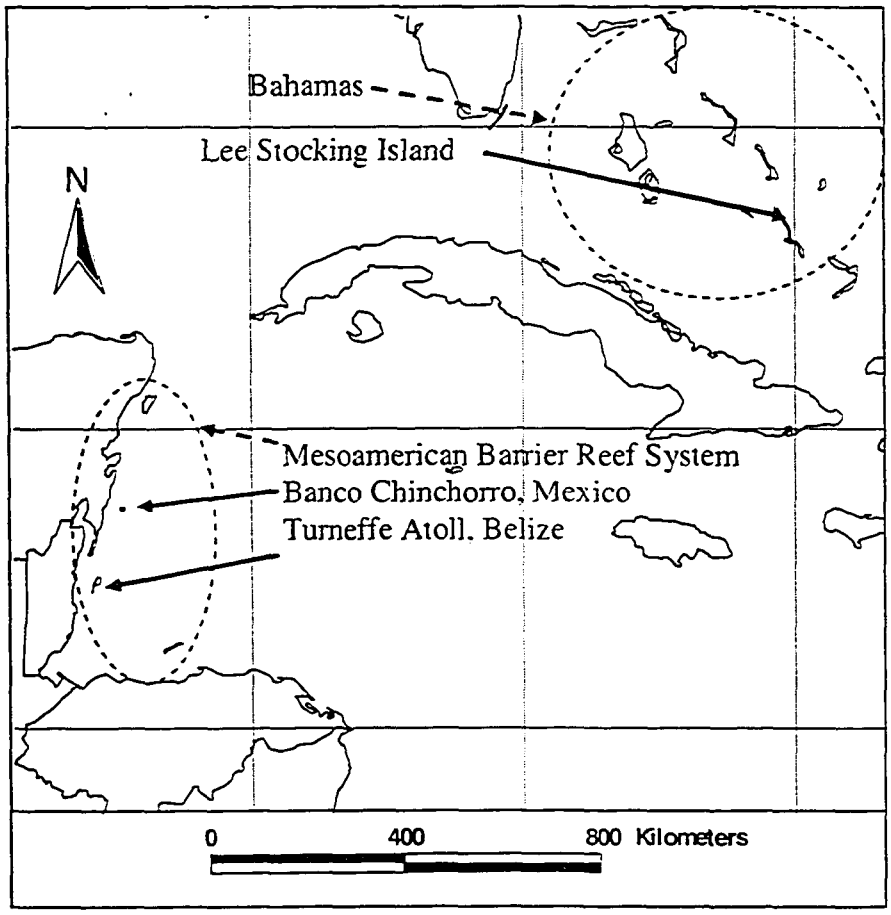


Figure 1.2: Maps of study regions (Belize, Mexico, and Bahamas) and locations (Turneffe Atoll, Banco Chinchorro, and Lee Stocking Island, respectively). Ovals represent location of regions.



Section A: Pelagic stage connectivity

Chapter 2: Discrimination of *Stegastes partitus* populations and an assessment of the movement of individuals at Turneffe Atoll, Belize.

A modified version of this chapter was submitted to Marine Ecology Progress Series with the following authors:

P.M. Chittaro, C. Mora, B.J. Fryer, and P.F. Sale.

Abstract

We examined the utility of otolith trace elemental chemistry of newly settled *Stegastes partitus*, as a means of discriminating populations and assessing their interconnectedness. Based on laser ablated otolith edges, results of a nested MANCOVA (fish standard length as covariate) indicated significant temporal (between years, among sampling periods within years) and spatial (among sites within sampling periods within years) variability in the concentrations of Li, Mg, Mn, Zn, and Ba.

To assess whether elemental variability (using otolith edge chemistry) was sufficient to differentiate sites at a given time (spatial analysis), and differentiate times for a given site (temporal analysis), separate discriminant function analyses (DFA) were performed. Results of the spatial analyses revealed a moderate degree of structure in microchemical signatures among sites, such that 50-69% (2002) and 54-66% (2003) of the individuals were correctly assigned to the site from which they were collected, while the temporal analyses indicated that microchemical signatures were variable at bimonthly scales, and thus individuals were correctly identified to the time they were collected an average of 83-94% (2002) and 60-81% (2003). The site from which individuals originated (i.e., the natal site), was estimated using the otolith edge chemistry as a chemical map of Tumeffe Atoll to which the otolith core chemistry (corresponding to natal origin) was compared. Results indicated that up to 1/3 of the individuals sampled (i.e., 6-35% of 48-58 individuals) had originated from one of the seven sites at Tumeffe Atoll.

Overall, these results indicate the utility of otolith microchemistry in distinguishing individuals collected from several sites and times as well as providing estimates of larval fish connectivity at an isolated location. Since the connectivity

investigation assumed a similar rate of trace element deposition into the otolith regardless of a fish's age or life history stage, future work should be directed to validate this approach and thus assess the accuracy of these estimates.

Introduction

Uncertainty about the extent of pelagic stage connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals: Mora and Sale 2002) highlights a significant gap in our knowledge of what determines the distribution and abundance of coral reef fish. Traditionally, reef fish larvae were regarded as passive particles since their relatively small body size was assumed to translate into a lack of directional control of their trajectory (Leis 2002). Consequently, the spatial extent of larval movement was believed to result from the combination of ocean currents and pelagic larval duration. As a result, populations were viewed as open, that is, they received larvae produced primarily by spawning from distant locations (Sale 1980; Williams et al. 1984; Doherty and Williams 1988; Sale 1991). However, evidence has indicated that some reef fish populations are relatively closed; that is, individuals hatched from a given area remain nearby or return to the population at the completion of larval life (often referred to as self-recruitment or local retention). For example, using the microstructure and microchemistry of otoliths (calcium carbonate structures found within most fish) of tropical reef fish, Jones et al. (1999) and Swearer et al. (1999) observed that self-recruitment occurred and thus the potential existed for populations to be closed.

Unfortunately, since the importance of self-recruitment to the maintenance of populations has only been empirically emphasized by these two studies (but see predictions based on hydrodynamic models by Black 1988, Black et al. 1990; Black and Moran 1991; Black et al. 1991; Black 1993; Cowen et al. 2000), an assessment is required of other regions that have a greater number of up-current sources, to test the general importance of self-recruitment in the maintenance of populations (Swearer et al. 2002). Mora and Sale (2002) have cautioned against hasty generalizations about the

extent of connectivity of reef fish populations, since this will depend upon the location (each having unique hydrography), the species (each having unique pelagic larval duration and behavioral capabilities), and the spatial and temporal scale at which the system is examined. Clearly, additional studies need to be conducted to assess fish dispersal and population connectivity.

Traditionally, tagging studies have been used to identify the movements of juvenile and adult fish (e.g., Nicholson 1978; Buckley et al. 1994; Jagielo 1990; Yamanaka and Richards 1993) and only rarely for larvae. For instance, Jones et al. (1999) tagged approximately 10 million embryos of *Pomacentrus amboinensis* (Ambon damselfish) with a fluorescent dye at a number of sites around Lizard Island, Great Barrier Reef, Australia. From a subsequent collection of 5000 larvae, 15 were found to contain the tag; based on the number of spawning adults and their fecundity, 15-60% of recruits were estimated to have been produced locally (i.e., 40-85% produced elsewhere). Larval tagging studies are often demanding projects, both financially and logistically, that require extensive marking programs since most marine fish are characterized by the mass production of pelagic larvae that experience high mortality (Thorrold et al. 2001; Hamer et al. 2003). Consequently alternative methods are sought that permit the identification of the movement of individuals among populations.

The microchemical nature of otoliths has been increasing used to assess the movement of individuals, since they provide a means to naturally tag individuals. Specifically, otolith microchemical studies takes advantage of the deposition of trace elements from the environment onto the growing surface of otoliths, which are at concentrations that reflect that of the environment (e.g., Sr- Farrell and Campana 1996;

Gallahar and Kingsford 1996; Bath et al. 2000; Milton and Chenery 2001; Kennedy et al. 2002; Elsdon and Gillanders 2003; Ba- Bath et al. 2000; Milton and Chenery 2001; Elsdon and Gillanders 2003; Li- Milton and Chenery 2001; Pb- Geffen et al. 1998). This otolith growth together with the deposition of elements allows the retrospective determination of site-specific chemical signatures and patterns of movement (Campana 1999), much like a flight recorder can be used to reconstruct an airplane's flight history. The microchemistry of otoliths has been used to investigate population structure (i.e., the discrimination of fish to populations of similar chemistry) and connectivity. For example, using differences in growth rate and trace-element concentrations in otoliths, Swearer et al. (1999) determined that 44.5% of the recruitment of *Thalassoma bifasciatum* (Bluehead wrasse) larvae to St. Croix, U.S.Virgin Islands, had developed within coastal waters and thus were the result of self-recruitment (i.e., locally spawned). Thorrold et al. (1998) used elemental concentrations of trace elements and stable isotopes to classify juvenile *Cynoscion regalis*, weakfish, to their natal estuary with a 60-70% success rate. Later, by examining adults spawned during the initial collection, they identified that 60-81% of individuals that were analysed showed signs of site fidelity (i.e., adults returned to the estuary in which they were spawned) (Thorrold et al. 2001).

Using otolith microchemistry to successfully discriminate individuals and assess the movement of individuals (i.e., assign fish to the site from which they originated), first requires a detectable level of chemical variability at biologically relevant spatial scales (Hamer et al. 2003) and temporal consistency such that site-specific chemical signatures do not need to be determined repeatedly (Gillanders 2002). Here we use otolith chemistry of a common Caribbean reef fish, *Stegastes partitus*, bicolor damselfish, to

investigate issues of population structure and connectivity. The objectives of this study were to use otolith chemistry from *S. partitus* collected from Turneffe Atoll, Belize, to a) assess whether populations can be discriminated, and if so, at what spatial and temporal scales, and b) to quantify to what degree, if any, sampled individuals originate from sites within Turneffe Atoll.

Materials and methods

To assess population discrimination and connectivity using otolith microchemistry, this study used *S. partitus*, Bicolor damselfish, a common Caribbean coral reef fish. *Stegastes partitus* (Class Actinopterygii, Order Perciformes, Family Pomacentridae) is a relatively small species (maximum total length of approximately 10 cm), common on shallow coral reefs throughout the Caribbean. They are demersal spawners whose pelagic larval stage is 29 to 35 days long (Robertson et al. 1988; Wellington and Victor 1989; Wilson and Meekan 2002), after which they settle to a reef habitat where they occupy a relatively small territory for the remainder of their lives. *Stegastes partitus* were chosen for the study because they are very abundant and relatively easy to catch with hand nets and clove oil. Furthermore, because of their relatively sedentary post-settlement existence, *S. partitus* otolith chemistry will be composed of the elemental history for a specific area, without any confounding effect that could be related to movement, if larger, more mobile species were studied.

Work was conducted at seven permanent sites (each site encompassed a total area of approximately 0.040 km^2, and each site was in approximately 10 m of water) surrounding Turneffe Atoll, Belize, in the western Caribbean, during the summers of 2002 and 2003 (Figure 2.1). Each site was visited consecutively every other week

(referred to as sampling period) centered on new and full moons, starting in late May and ending in late August for both 2002 and 2003 (Table 2.1). At each site, a minimum of 10 newly settled *S. partitus* (defined here as < 2.5 cm total length) were collected using hand nets and clove oil and stored in 95% ethanol.

Sagittae from fish collected at sampling periods 1, 2, 3, 5, and 7 (Table 2.1) were removed, cleaned of tissues, embedded in Crystal Bond®, and polished in a transverse plane using 30-3 μm lapping film. In a class 100 clean room, multiple otolith sections (up to 35) were fixed to a microscope slide and cleaned by placing them first in a milliQ water bath that was sonicated for 2.5 minutes, triple rinsed in 95% ethanol, and triple rinsed in milliQ water. Each slide was then allowed to dry overnight in a laminar flow HEPA-filtered fume hood.

Otoliths were chemically analyzed at the Great Lakes Institute of Environmental Research, University of Windsor, using laser ablation inductively coupled plasma mass spectrometry (LA-IC-PMS). A Thermo Elemental X7 ICP-MS was operated at low resolution with argon used as the carrier gas from the laser-sampling cell. The laser sampling system is a purpose-built system (Fryer et al. 1995) based on a non-homogenized, high power, frequency-quadrupled (266 nm) Nd:YAG (neodymium-doped yttrium aluminum garnet) laser. Frequency was set at 20 Hz, flash lamp power was 1.15 kV, and a 1 mm pinhole beam constrictor was used to decrease the spatial resolution of the laser sampling. Based on these settings, laser ablation diameter and depth were approximately 15 and 45 μm, respectively. The laser beam was focused onto the sample using an Olympus® BX-51 petrographic microscope and an Optics For Research® 266

nm 10 X objective lens. The sampling system is more fully described in Crowe et al. (2003).

LA-IC-PMS data acquisition lasted 140 seconds with 60 seconds of instrument and gas background counts prior to the start of each ablation. Transects from the otolith core to edge (i.e., perpendicular to growth increments) were targeted using an automated microscope stage moving at a speed of approximately 5 μm/sec. A trace element-doped glass standard (National Institute of Standards and Technology, NIST, 610, a homogenous glass that is spiked with a range of elements of known concentrations) was analyzed in duplicate at the beginning and end of each sample set (20 samples) to correct for instrument drift. Calcium was used as an internal standard to compensate for signal variation caused by differences in mass of ablated material.

In total, 13 elements were analyzed by the LA-ICP-MS and chemical concentrations (parts per million) were calculated using Lamtrace software (van Achterbergh et al. 2001). The elemental signal selected for use in Lamtrace was limited to a 20μm length at the core and edge of each otolith (the 20 μm length is based on the average diameter of pre-hatch *S. partitus* embryos, see Chapter 4, and corresponds to approximately 2-3 days growth, based on estimates of settlement stage fish by Wilson and Meekan 2002). The otolith core chemistry is presumed to correspond to the space and time an individual was hatched, while the otolith edge chemistry represents the space and time it was collected. After signal selection, elements that met the following two criteria were included in statistical analyses; 1) concentrations of NIST samples were determined with a satisfactory precision (coefficient of variation less than 10%); and 2) concentrations in otoliths were greater than detection limit for more than 50% of otoliths

analyzed. For elements meeting the above criteria, outlier analysis was performed for each element such that any value that was three times the interquartile distance was removed (see Fowler et al.1995; StatSoft 2001). Unless otherwise reported, data were $\log_{10}$ transformed to improve normality for multivariate analyses (see below).

To investigate whether otolith chemistry varied significantly through space and time, we applied a multivariate approach using otolith edge elemental concentrations. The multivariate statistics required tests of homogeneity of slopes (this assumption is necessary when incorporating a covariate into an analysis), homogeneity of variance-covariance, normality, linearity, and multicollinearity. If assumptions were met, then a nested MANCOVA was used with sites (7 sites) nested within sampling periods (i.e., 1, 2, 3, 5, and 7), which were nested within years (2002 and 2003). Since trace element incorporation may be related to growth, and thus to the size and age of fish (Begg et al. 1998), the interpretation of spatial and/or temporal variation may be confounded if fish are of different ages and/or sizes. To reduce any influence of fish size, fish standard length was used as a covariate. Dependent variables were the elemental concentrations and independent variables were site, sampling period, and year.

If the MANCOVA (which is based on the otolith edge elemental concentrations) revealed significant results, then direct discriminant function analysis (DFA) (which maximizes the among-group variation relative to the within group variation; McCune and Grace 2002) was used to determine if fish could be accurately classified to the site or sampling period from which they were collected, and the element(s) that accounted for this separation. Specically, to investigate spatial variability in otolith chemistry (i.e., the spatial analysis), separate DFAs were performed comparing sites within each of the five

sampling periods (i.e., sampling periods 1, 2, 3, 5, and 7) in 2002 and 2003. To investigate temporal variability in otolith chemistry (i.e., the temporal analysis), separate DFAs were performed comparing sampling periods within each of the seven sites.

For each DFA, a classification matrix and partial Wilks' Lambda statistic was determined: the former indicates the percent of fish that were correctly identified to the site or sampling period from which they were collected, while the latter indicates the unique contribution of an element to the discriminatory power of the model (StatSoft, Inc. 2001). To interpret which element best accounted for the separation within a discriminant root, factor correlations greater than |0.33| (representing approximately 10% of the variance; Tabachnick and Fidell 1996) were examined.

To investigate patterns of movement of individuals among sites within Tumeffe Atoll (i.e., the connectivity analysis) we used the predictive capabilities of DFA. Specifically, we used the spatial DFA of otolith edge chemistry (for a given year and sampling period) as the training dataset to generate a chemical map and produce classification functions. We then used these classifications functions to estimate the natal sites from which individuals originated based on the test dataset of otolith core chemistry. To assess the reliability of these classifications, *posterior probabilities* were examined for the training and test data sets. *Posterior probabilities* are determined from mahalanobis distances (the distance of each case to the mean centroid of the nearest group; McCune and Grace 2002) and *a priori* classifications (for our purposes *a priori* classifications were proportional to sample size), and represent the probability of a fish belonging to a given site. A greater *posterior probability* means a higher confidence in the assignment of an individual to a particular site.

To ensure confidence in our estimates of connectivity, we interpreted connectivity only for those individuals of the test dataset that had *posterior probabilities* > 90%. However, even though an individual may have a high *posterior probability*, there is a possibility that it is incorrectly classified. To assess this kind of error (an incorrect classification with high confidence), we examined the degree to which individuals from the training set that were assigned with high confidence (i.e., >90% posterior probability) were misidentified. The number of misidentified individuals and the distance associated with this error (i.e., the linear distance between the predicted site and the known site of collection) were used to help refine and interpret patterns of connectivity.

Results

The average number of otoliths analyzed per site and sampling period was 7 for 2002 (+/-4; n=280) and 8 for 2003 (+/-2; n=311). Following outlier analysis, 46 individuals were removed from both years, and therefore 234 and 265 otoliths were statistically examined from 2002 and 2003, respectively. Based on the two criteria (coefficient of variation less than 10% for NIST samples and concentrations in otoliths greater than detection limit for more than 50% of otoliths analyzed) seven elements (Li, Mg, Mn, Zn, Sr, Ba, and Pb) were included for statistical analyses in 2002 and 2003, with the addition of Ce in 2003 (Table 2.2).

Since the assumption of homogeneity of slopes was not met (i.e., there was a significant interaction between sites or sampling periods and the covariate, with respect to elemental concentrations), a separate slopes nested MANCOVA was performed. From this analysis, the multivariate test indicated significant differences in otolith elemental

concentrations at each level of the design: between years (Wilks' Lambda =0.17; $F_{7,423}$=293; p<0.001), among sampling periods within years (Wilks' Lambda =0.15; $F_{56,2283}$=17; p<0.001), and among sites within sampling periods and years (Wilks' Lambda =0.14; $F_{413,2956}$=2.32; p<0.001). Inspection of the univariate analyses indicated significant differences at each level of the design for Li, Mg, Mn, Zn, and Ba, while Sr and Pb did not show significant variability among years (Table 2.3). Concentrations of Li, Mg, Mn, and Ba were lower and more variable in 2002 compared to 2003, yet concentrations of these elements increased in 2002 across sampling periods, but were relatively constant in 2003 (Pb appeared to decrease slightly through time in 2003) (Figure 2.2a and b). A spatial pattern in concentrations (determined from Tukey's HSD posthoc test for unequal sample sizes) was only observed for Li, in both years, since otoliths from sites 6 and 7 (northwest side of Tumeffe Atoll) had consistently the lowest concentrations, while those from sites 1 and 2 (northeast side) had the highest concentrations.

Spatial analysis

DFA of fish among the seven sites in 2002 indicated significant discrimination for all sampling periods (Table 2.4). Across all of the spatial analyses, the first two functions explained 51-89% of the variation (sampling periods 2 and 7 had only one significant discrimination function) and as a result fish were correctly identified to the site from which they were collected with 50-69% average accuracy (Table 2.4). Of 234 fish analyzed for 2002, 84 individuals were misidentified, of which 38% (i.e., 32 individuals) were classified to adjacent sites, suggesting a relatively small degree of spatial correlation

in otolith microchemistry at the scale of 5-20 kms. For each spatial DFA of 2002, the partial Wilks' Lambda indicated that Li was the most important trace element in the separation of sites.

DFA of fish among the seven sites in 2003 indicated significant discrimination for all sampling periods, albeit slightly reduced discrimination relative to 2002 (Table 2.4). Across all of the spatial analyses the first two functions explained 49-75% of the variation (sampling periods 3 and 5 had only one significant discriminant function) and as a result fish were correctly identified to the site from which they were collected with 54-66% average accuracy (Table 2.4). Of 265 fish analyzed for 2003, 104 individuals were misidentified, of which 34% (35 individuals) were classified to adjacent sites suggesting a similar level of spatial consistency in otolith microchemistry relative to 2002. Of the five spatial DFAs of 2003, Li explained the greatest amount of variability (i.e., discriminatory power) for three sampling periods (1, 3, and 5) while Zn was most important for sampling period 2 and Mn for sampling period 7.

Temporal analysis

DFA of fish among the five sampling periods of 2002 (ranging from 2 to 4 weeks separating consecutive sampling periods), for each of the seven sites, indicated substantial temporal variability that resulted in significant discrimination of individuals (Table 2.4). Across all of the temporal analyses, the first two functions explained 91-96% of the variation (site 6 showed a lack of significant discrimination) and as a result fish were correctly identified to the sampling period they were collected with 83-94% average accuracy (Table 2.4). Of 206 fish analyzed for 2002, 23 individuals were

misidentified, of which 48% (11 individuals) were classified to the adjacent time period suggesting some degree of temporal consistency in otolith microchemistry at the time scales of this investigation. In fact, 4 of the 11 individuals that were misclassified were from sampling periods 2 and 3, which were temporally separated by 2 weeks. For each temporal DFA of 2002, otolith chemistry from 3 sites (3, 4, and 7) indicated that Li explained the greatest amount of variability, while Mg (site 5), Pb (site 1) and Mn (site 2) were each important to one site.

DFA of fish among the 5 sampling periods of 2003, for each of the 7 sites, indicated temporal variability that was less variable than 2002, yet still resulted in significant discrimination in the 7 analyses (Table 2.4). Across all temporal analyses the first function explained 53-78% of the variation (site 7 had two significant discriminant functions with 83% of the variation explained) and as a result fish were correctly identified to the sampling period they were collected from with 60-81% average accuracy (Table 2.4). Of 265 analyzed fish for 2003, 81 were misidentified, of which 48% (i.e., 39 individuals) were classified to the nearest sampling period suggesting a similar level of temporal consistency in otolith microchemistry relative to 2002. Of the temporal DFAs of 2003, Zn (sites 2 and 7) and Pb (sites 5 and 6) explained the greatest amount of discrimination, while Mg (site 3), Mn (site 4), and Sr (site 1) were each important to one site.

Connectivity analysis

The lack of temporal stability in otolith chemistry (on a two week scale, see above) meant that comparisons between the chemical map (determined from otolith

edges) and the otolith cores were inappropriate unless these portions of the otolith were deposited at the same time. Since the pelagic larval duration for *S. partitus* is between 29 and 35 days (Robertson et al. 1988; Wellington and Victor 1989; Wilson and Meekan 2002), we needed to compare the otolith edge chemistry of individuals to the otolith core chemistry of those collected one-month later. Fortunately, we had collections of *S. partitus* that were separated by one-month (+/- four days). Therefore, three separate investigations of connectivity were possible within each year; edge chemistry from sampling periods 1, 3, and 5 were the training datasets that were used to derive the classification functions to identify the site of origin for individuals collected from sampling periods 3, 5, and 7, respectively, whose otolith core chemistry was determined.

The investigation of the relative contribution of fish spawned from within Tumeffe Atoll, revealed that across six connectivity investigations, 6-35% (mean 17% and standard deviation 11%) of the individuals sampled (of 48-58 individuals) were classified (based on >90% posterior probabilities) to one of seven sites. Specifically, across both summers (2002 and 2003), 19, 4, 6, 5, 0, 11, and 7 individuals were found to have originated from sites 1, 2, 3, 4, 5, 6, and 7, respectively, and 9, 8, 12, 2, 9, 3, and 9 individuals were collected from sites 1, 2, 3, 4, 5, 6, and 7, respectively (Figure 2.3). Based on these estimates of connectivity, the northernmost site (site 1) appeared to be the source for many individuals collected elsewhere (most of which occurred during a single sampling period, Figure 2.3c) while the southernmost site (site 5) was not found to be the source of any individuals.

For 2002 analyses, and in particular the training set of sampling period 1, one individual (out of 11 with >90% posterior probability) was incorrectly classified even

though it had a high posterior probability (>90% posterior probability). The linear distance associated with this error was 28.8 km (i.e., the distance between the actual and the predicted site). Based on this training data set, 11 individuals (of 49) from the test data set (i.e., core otolith chemistry of sampling period 3) were classified (with >90% posterior probability) to six of the seven sites (Figure 2.3a). The linear distance traveled ranged from 0 km (settlement to the same site from which it was estimated to have originated) to 36 km. However, because of the observed error rate (one individual out of 11 were incorrectly classified although confidence was high), it is likely that some (at least 1/11) of these connectivity estimates are incorrect.

For the training set of sampling period 3, no individual with a posterior probability >90% was incorrectly classified. Based on this training data set, 4 individuals (out of 50) from the test data set (i.e., core otolith chemistry of sampling period 5) were classified (with >90% posterior probability) to three of the seven sites (Figure 2.3b). The linear distance traveled ranged from 0 km to 24 km.

For the training set of sampling period 5, no individual with a posterior probability >90% was incorrectly classified. Based on this training data set, 17 individuals (out of 48) from the test data set (i.e., core otolith chemistry of sampling period 7) were classified (with >90% posterior probability) to two of the seven sites (Figure 2.3c). A distinct southward dispersal pattern was observed for this sampling period. The linear distance traveled ranged from 0 km to 48 km.

For all three 2003 analyses, no individual with a posterior probability >90% was incorrectly classified. Based on the training set of sampling period 1, 6 individuals (out of 58) from the test data set (i.e., core otolith chemistry of sampling period 3) were

classified (with >90% posterior probability) to two of the seven sites (Figure 2.3d). The linear distance traveled ranged from 0 km to 29 km.

For the training set of sampling period 3, 11 individuals (out of 48) from the test data set (i.e., core otolith chemistry of sampling period 5) were classified (with >90% posterior probability) to two of the seven sites (Figure 2.3e). The linear distance traveled ranged from 0 km (settlement to the same site from which it was estimated to have originated) to 35 km.

For the training set of sampling period 5, 3 individuals (out of 53) from the test data set (i.e., core otolith chemistry of sampling period 7) were classified (with >90% posterior probability) to two of the seven sites (Figure 2.3f). The linear distance traveled ranged from 14 km to 20 km.

Discussion

The spatial variability in otolith trace elemental concentrations of *S. partitus* among sites separated by 5 to 49 km, and within a location (Turneffe Atoll) that is relatively isolated from coastal influences, indicated some overlap in otolith chemical signatures that resulted in moderately accurate discriminatory ability. Previous studies have detected sufficient variability in otolith chemistry to discriminate fish collected from sites separated by distances of 10-600 km (e.g., see work on temperate species by Edmunds et al. 1991; Campana et al. 1994; Volk et al. 2000; Thorrold et al. 2001; Forrester and Swearer 2002; Rooker et al. 2003; Swearer et al. 2003; and Wells et al. 2003; and on tropical species by Edmonds et al. 1989; Sadovy and Severin 1992 and 1994; Dufour et al. 1998; Milton and Chenery 2001, 2003; Bastow et al. 2002). Yet most of this work was conducted in coastal locations that are characterized by relatively more

spatially variable chemical signatures resulting from inputs of terrigenous sediments and anthropogenically derived pollutants, compared to that of isolated locations (Thorrold et al. 2002), such as Tumeffe Atoll. Hamer et al. (2003) identified substantial variability in otolith microchemistry of *Pagrus auratus* (Snapper) collected from several sites (separated by < 10 km) in multiple bays (100s of km) along the Victorian coast of Australia. However, studies conducted in locations more isolated from coastline-derived influences have had limited success discriminating populations. For instance, the otolith chemistry of *Epinephelus striatus* (Nassau grouper) collected from three relatively isolated sites (separated by 40-70 km) in the Exuma Sound of Bahamas, did not differ significantly, while differences were detected when fish from Bahamas and Belize (sites separated by approximately 1500 km) were compared (Patterson et al. 1999). Similarly, along the Great Barrier Reef (GBR), Australia, Patterson et al. (2004) observed significant spatial variability in otolith microchemistry of *Pomacentrus coelestis* (Neon damselfish) between northern and southern latitudes of the GBR (separated by 1100km), but not among reefs within latitudes (separated by approximately 4-25 km). In comparison, we detected variable elemental signatures among collections of *S. partitus*, which enabled individuals from sites as close as 5 km to be discriminated with reasonable accuracy.

This discriminatory ability was surprising considering that Tumeffe Atoll is a relatively isolated location (approximately 35 km from the mainland), with little development (4 resorts and 1 research station, which have a maximum occupancy ranging from 20 to 75 people) (Figure 2.1), and relatively few, if any, sources of freshwater input. However, the elemental variability found in the otoliths of reef fish

from Turneffe Atoll may be influenced by the decomposition of detritus from the large expanse of seagrass and mangrove habitats, the shallow, warm, and relatively stagnant waters in which these habitats thrive, and the tidal fluxes that may transport these lagoonal waters to fish on nearby reefs. Regardless of the mechanism, the moderate degree of chemical variability at such small spatial scales highlights the potential for assigning fish to sites from which they originated. However, an understanding of the temporal variability in otolith microchemistry is also required before otolith microchemistry can be used to determine where fish originated.

Because of temporal variability in otolith microchemistry, individuals collected at the same site, but two weeks, apart were easily separated. To our knowledge, this is the first study to observe such temporal variability in otolith chemistry at this scale (see Table 4 in Gillanders 2002). Kalish (1989) and Hamer et al. (2003) noted significant variability in otolith chemistry of *Arripis trutta* (Australian salmon) and *Pagrus auratus* (Pink snapper), respectively between samples separated by four weeks. Swearer et al. (2003) observed variability in otolith microchemical signatures after five months for each of three species (*Atherinops affinis*, *Paralichthys californicus*, and *Clevelandia ios*), while Patterson et al. (2004) noted variability after one year for *Pomacentrus coelestis* (within year comparisons were not made). Thorrold et al. (1998) observed consistency in the otolith signatures of *Alosa sapidissima* (American shad) at three months, while Campana et al. (2000), indicated consistency up to one year (but not at 4–13 years) in Atlantic cod (*Gadus morhua*). Consequently, both of these latter studies suggested, contrary to what the otolith edge chemistry of *S. partitus* indicated, that otolith chemistry would serve as a seasonal biological tracer of known groups of fish. The substantial

temporal variability in otolith microchemistry observed in *S. partitus* at Turneffe Atoll, complicates the assessment of patterns of connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals: Mora and Sale 2002) since it will require that only the otolith chemistry from fish collected at specific times be used. *In other words, to investigate patterns of connectivity, we need to* compare the natal chemistry (i.e., otolith core chemistry) to the edge chemistry that was formed at the same time.

Using trace elemental concentrations derived from the natal portion of *S. partitus* otoliths, and comparing them to the appropriate collection of individuals and their edge chemistry, resulted in the observation that several individuals collected from Turneffe Atoll sites originated from within Turneffe Atoll. Specifically, within any of the six sampling periods of 2002 and 2003, in which we investigated the dispersal of reef fish, we estimated that 3-17 individuals (out of 48-58 individuals) originated from within Turneffe Atoll. Based on these individuals and their predicted dispersal pathways, dispersal distances ranged from 0 to 48 km (i.e., linear distances between sites and thus not the likely path taken by pelagic larvae). Just over 40% of these individuals were observed to disperse approximately 20 km from their site of hatching, while 17% showed no dispersal at all (Figure 2.4). Because of the lack of confidence in the classification of the remaining individuals (i.e., individuals with posterior probabilities <90%), we avoided speculating on their origins. It is possible that some of these individuals would have been found to originate from within Turneffe Atoll if a finer spatial scale of otolith edge chemistry had been used (i.e., if the otolith microchemistry was investigated of fish collected from more sites).

Although we obtained estimates of pelagic larval stage connectivity, there was no means to assess its accuracy. Unlike the mark-recapture technique employed by Jones et al. (1999) that provided definitive evidence of self-recruitment, this study made several assumptions in order to investigate connectivity through the comparison of the natal (core) and demersal (edge) chemistry, and thus a degree of caution is necessary when interpreting our results. For instance, since the core corresponds to a period of embryonic development, and thus a stage of substantial physiological and morphological change, the deposition of trace elements into an otoliths core may not necessarily reflect the environment to the same degree as other life history stages (see Kalish 1989, 1991; Toole et al. 1993; Otake et al. 1994; Fowler et al. 1995; de Pontual and Geffen 2002). Consequently, estimates of connectivity may be incorrect since differences in elemental concentrations may occur between otolith core and edge chemistry even for fish whose origin and settlement, respectively, were from the same site (see Chapter 4 for an investigation of ontogenetic variability in otolith chemistry). In addition, otolith crystal structure and otolith growth rate can influence the deposition of trace elements and may vary with life history stage (Campana and Gagne 1995; Gauldie 1996; Brown and Severin 1999; Brophy et al. 2004; Patterson et al. 2004) further complicating the comparison of otolith edge and core chemistry (see Chapters 3 and 4 for more detail).

In conclusion, this study has shown that significant variability in otolith microchemistry of *S. partitus* exists among sites throughout Turneffe Atoll, Belize, and among repeat samples in both 2002 and 2003. According to this study, the minimum spatial and temporal scales that permitted accurate discrimination of individuals, was 5 km and two weeks, respectively. Due to the temporal variability observed in the otolith

edge chemistry of successive collections of *S. partitus*, the use of otolith core chemistry to discern sources of individuals required the spatial chemistry determined from the otolith edge of individuals collected at the corresponding period. Based on these otolith core-edge comparisons, up to 1/3 of the individuals (i.e., 3 to17 out of 48-58 individuals) sampled at any given time were estimated to have originated from within Turneffe Atoll. Some or all of the remaining individuals may have arrived from further away. The results of this study highlight the value of using otolith microchemistry to discriminate populations and assess their interconnectivity, even at isolated locations like Turneffe Atoll. However, because of assumptions regarding the deposition of trace elements within fish of different life history stages, future work must validate this microchemical approach.

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Table 2.1: Dates of sampling periods in which newly settled *S. partitus* were collected from sites at Turneffe Atoll, Belize.

Year	Sampling period	Date
2002	1	May 29-June 3
	2	June 14-17
	3	June 25-July 3
	4	
	5	July 22-28
	6	
	7	August 23-25
2003	1	June 1-7
	2	June 15-21
	3	June 30-July 6
	4	
	5	July 30-August 5
	6	
	7 *	August 28-31

* only 6 sites sampled.

Table 2.2: Elements included in statistical analyses that were determined from the coefficient of variation (CV) of NIST samples being < 10% and > 50% of otolith samples with values greater than detection limit. Average and standard deviation (SD) provided for detection limit by sample ablation, overall concentration, and concentrations per year. Average CV is of 46 different sets of otolith ablations (20 and 26 for 2002 and 2003, respectively). All values are in parts per million.

Element measured (isotope)	Average CV (SD)	% of samples > detection limit	Average detection limit by sample (SD)	Overall average concentration (SD)	Average concentration 2002 (SD)	Average concentration 2003 (SD)
Sample size				499	234	265
Lithium (7)	5.29 (4.08)	99	0.08 (0.04)	0.85 (0.88)	0.38 (0.44)	1.27 (0.97)
Magnesium (24)	2.14 (1.13)	98	4.65 (2.69)	15.8 (12.1)	9.83 (6.85)	21.0 (13.4)
Manganese (55)	4.29 (2.56)	88	0.56 (0.45)	0.95 (0.72)	0.47 (0.39)	1.38 (0.69)
Zinc (66)	10.6 (11.6)	92	0.56 (0.77)	2.09 (2.22)	1.11 (1.08)	2.95 (2.58)
Strontium (86)	1.79 (1.22)	100	1.12 (0.79)	2902 (803)	3120 (996)	2709 (508)
Barium (138)	2.82 (1.34)	98	0.02 (0.10)	7.31 (6.20)	5.70 (4.22)	8.62 (7.24)
Cerium (140)*	2.58 (1.62)	91	0.01 (0.004)			0.17 (0.29)
Lead (208)	4.72 (2.60)	98	0.03 (0.05)	7.70 (24.6)	10.2 (34.9)	5.41 (6.13)

* indicates information only for 2003 samples.

Table 2.3: Univariate results of the nested MANCOVA, whereby site was nested within sampling period (SP) and year, while standard length (STDL) was the covariate.

Effect		Li				Mg				Mn				Zn			
	df	SS	MS	F	P<	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
Year	1	49	49	935	0.001	12	12	482	0.001	27	27	716	0.001	15	15	154	0.001
SP(Year)	8	33	4	78	0.001	7	0.9	35	0.001	10	1	34	0.001	8	1	10	0.001
Site (SP(Year))	59	16	0.3	5	0.001	2	0.04	1	0.01	4	0.07	2	0.001	12	0.2	2	0.001
STDL	1	2	2	42	0.001	0.2	0.2	8	0.01	0.001	0.001	0.02	NS	0.8	0.8	8	0.01
Error	429	23	0.05			10	0.02			16	0.03			43	0.09		

Effect		Sr				Ba				Pb			
	df	SS	MS	F	p<	SS	MS	F	P	SS	MS	F	p
Year	1	0.0006	0.0006	0.06	NS	8	8	104	0.001	0.1	0.1	0.9	NS
SP(Year)	8	0.3	0.03	4	0.001	7	0.9	12	0.001	32	4	25	0.001
Site (SP(Year))	59	0.9	0.01	2	0.01	7	0.1	2	0.01	27	0.5	3	0.001
STDL	1	0.5	0.5	55	0.001	5	5	67	0.001	0.04	0.04	0.3	NS
Error	429	4	0.01			32				68	0.2		

N.S. indicates non-significance (p>0.05).

Table 2.4: Results of the discriminant function analyses examining spatial and temporal variability in otolith edge microchemistry.

Analysis	Year	Within sampling period	Wilks' lambda	df	F	p <	% variance explained by 2 Roots	Chi - squared	df	p <	Average % correct	Range
Spatial	2002	1	0.06	42, 148	2.85	0.001	74	99	42	0.001	68	50-75
		2	0.13	42, 125	1.61	0.05	51 (1 root)	63	42	0.05	69	33-88
		3	0.13	42, 181	2.33	0.001	74	87	42	0.001	67	17-90
		5	0.04	42, 176	4.03	0.001	89	132	42	0.001	66	50-80
		7 +	0.28	35, 162	1.60	0.05	67 (1 root)	53	35	0.05	50	22-71
Spatial	2003	1	0.12	48, 205	2.34	0.001	72	99	48	0.001	66	44-78
		2	0.09	48, 181	2.27	0.001	75	96	48	0.001	64	42-100
		3	0.13	48, 215	2.31	0.001	62 (1 root)	99	48	0.001	54	38-71
		5	0.14	48, 176	1.78	0.01	49 (1 root)	78	48	0.01	59	42-75
		7	0.18	48, 200	1.72	0.01	65	77	48	0.01	57	25-75
		Within site										
Temporal	2002	1	0.03	28, 87	5.14	0.001	95	98	28	0.001	86	66-100
		2	0.01	28, 84	10.4	0.001	96	146	28	0.001	94	66-100
		3	0.04	28, 87	4.26	0.001	93	87	28	0.001	83	50-100
		4	0.03	28, 87	4.53	0.001	91	90	28	0.001	89	71-100
		5	0.01	28, 73	7.63	0.001	94	118	28	0.001	90	75-100
		6+	0.21	21, 52	1.72	NS		33	28	NS		
		7	0.02	28, 91	6.28	0.001	94	112	28	0.001	89	80-100
Temporal	2003	1	0.17	32, 93	1.79	0.05	67 (1 root)	52	32	0.05	73	50-78
		2	0.21	32, 97	1.62	0.05	53 (1 root)	48	32	0.05	66	33-89
		3	0.12	32, 90	2.16	0.01	60 (1 root)	60	32	0.01	64	28-100
		4	0.12	32, 97	2.35	0.001	78 (1 root)	64	32	0.001	60	25-100
		5	0.17	32, 97	1.89	0.01	71 (1 root)	54	32	0.01	66	55-88
		6	0.11	32, 108	2.78	0.001	69 (1 root)	74	32	0.001	76	60-88
		7	0.07	32, 93	3.13	0.001	83	79	32	0.001	81	60-100

NS indicates non-significance ($p > 0.05$).

+ Indicates that time period 7 of 2002 did not have fish for site 6.

Figure 2.1: Map of Turneffe Atoll, Belize, showing sites (filled circles) from which *S. partitus* were collected. Crosses indicate locations of development (in the form of resorts and one field station).

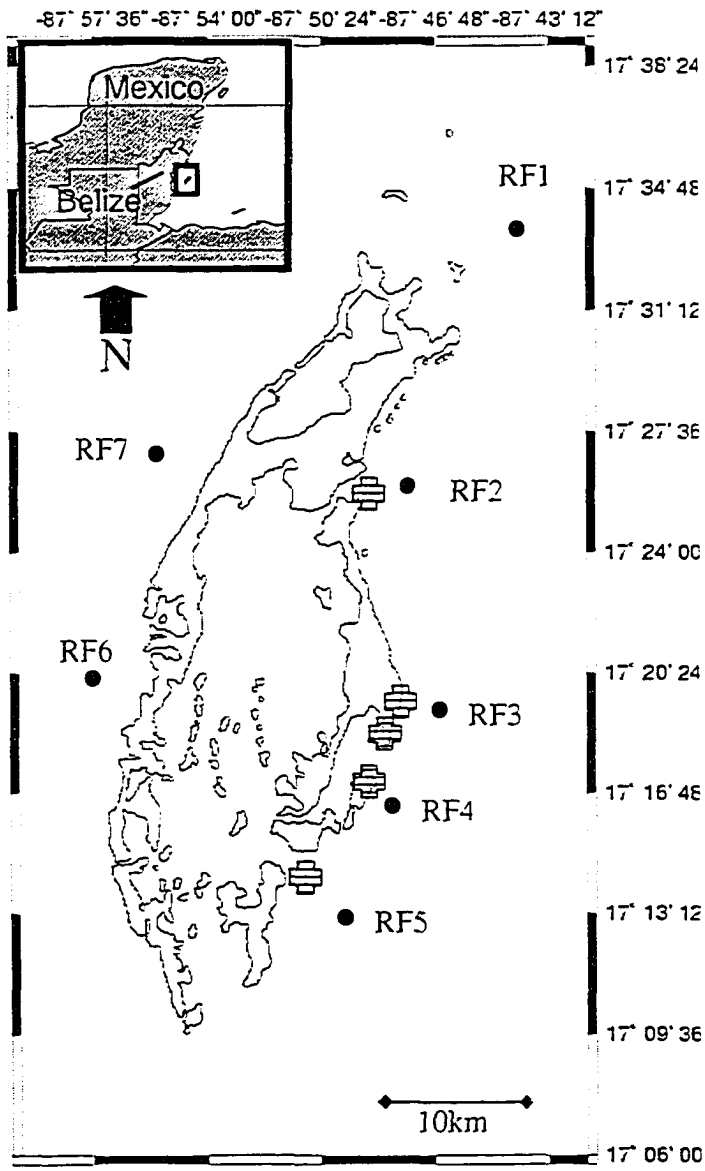


Figure 2.2a: Adjusted elemental concentrations (ppm) of otolith edges per site for each sampling period, and year. Means and 95% confidence limits are presented.

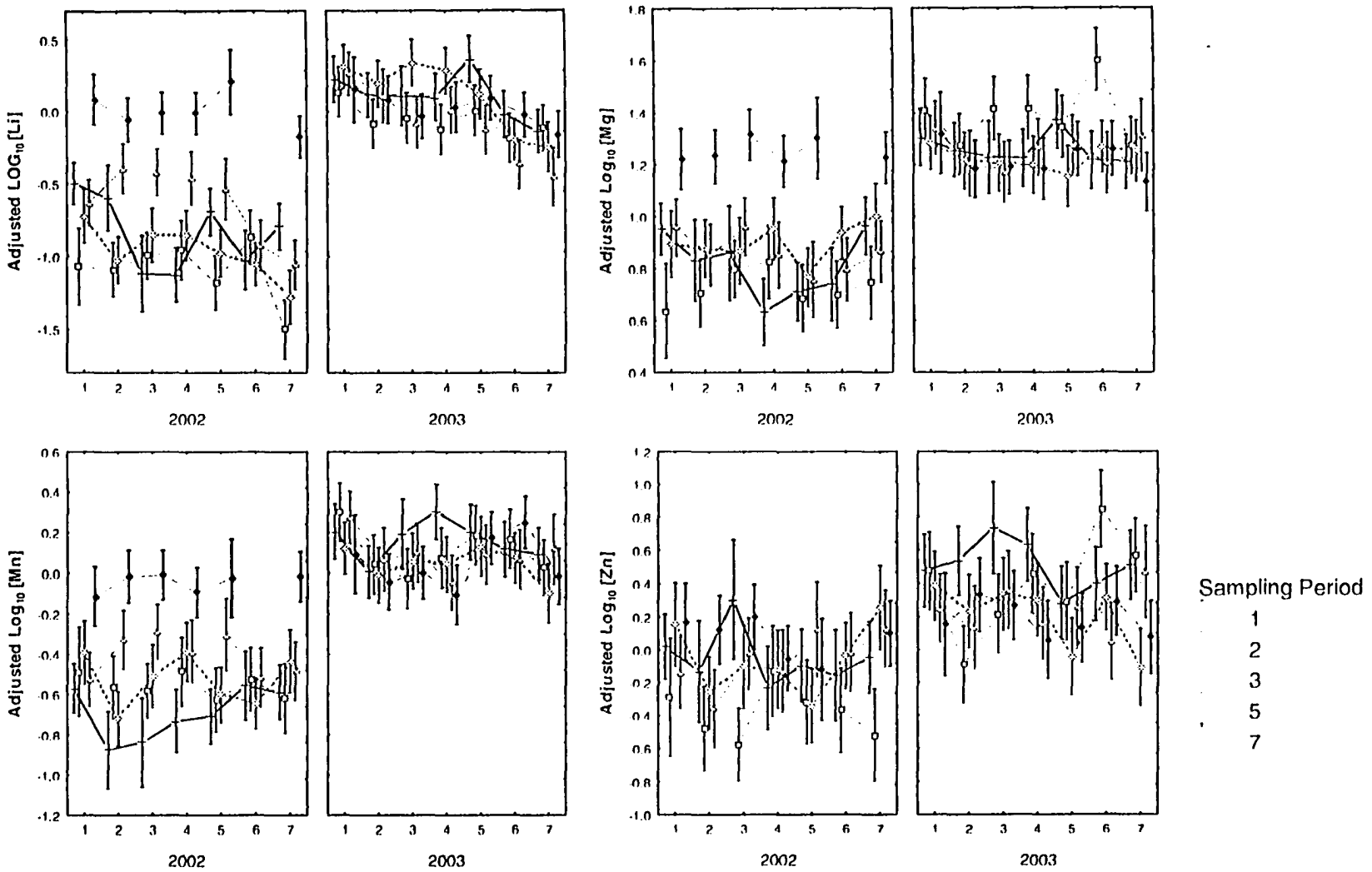


Figure 2.2b: Adjusted elemental concentrations (ppm) per site of otolith edges for each sampling period, and year. Means and 95% confidence limits are presented.

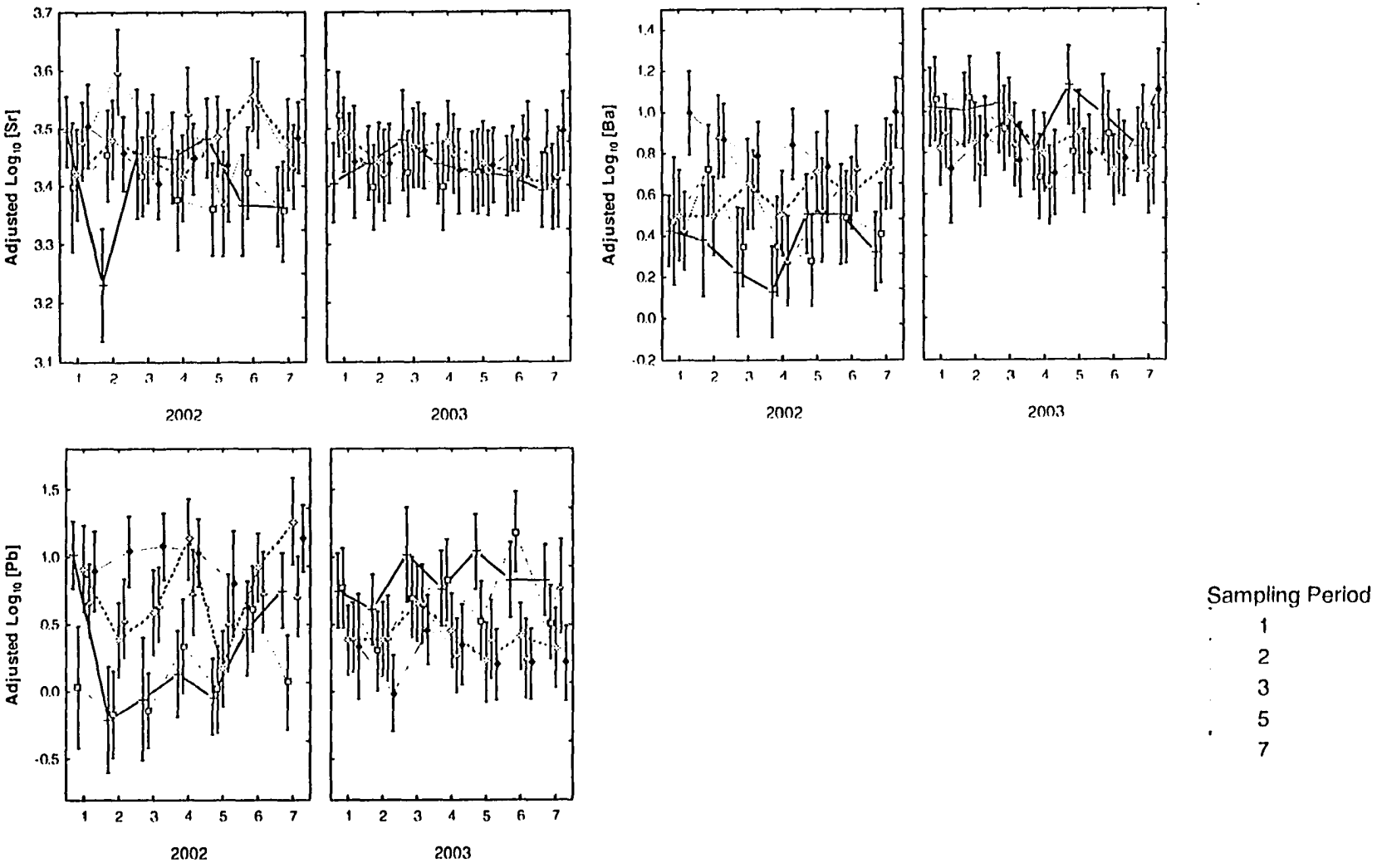


Figure 2.3: Patterns of larval dispersal among sites at Turneffe Atoll in 2002 sampling periods 1 (a), 3 (b) and 5 (c), and 2003 in sampling periods 1 (d), 3 (e), and 5 (f). A solid line indicates an individual's movement away from the site it was spawned to the site it was collected. A dashed line indicates that an individual settled where it was spawned. Each line (dashed or solid) represents the movement of one individual. Only those individuals from DFA of test data set that had posterior probabilities >90% are indicated. n denotes the number of individuals whose natal origin was estimated.

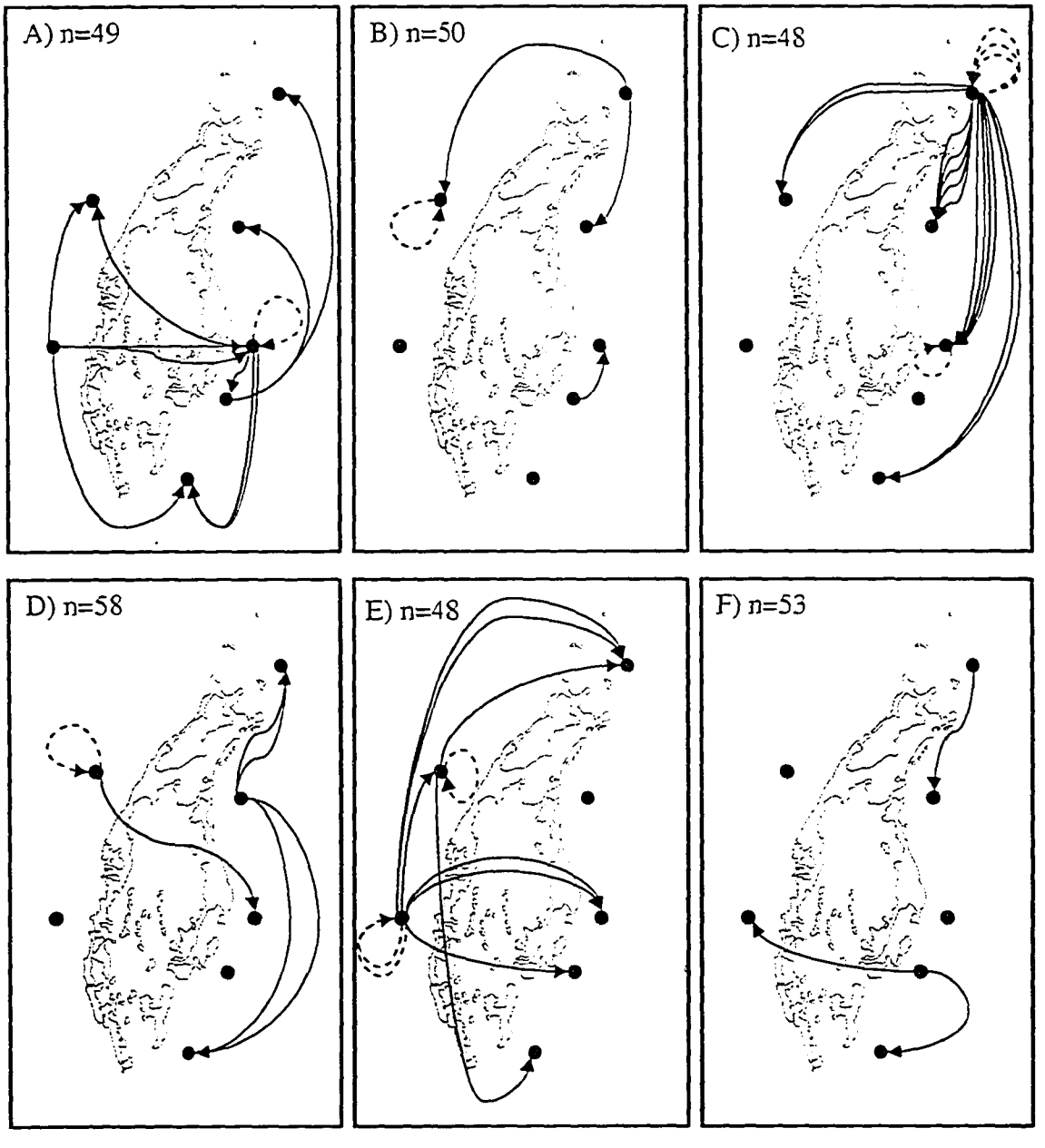
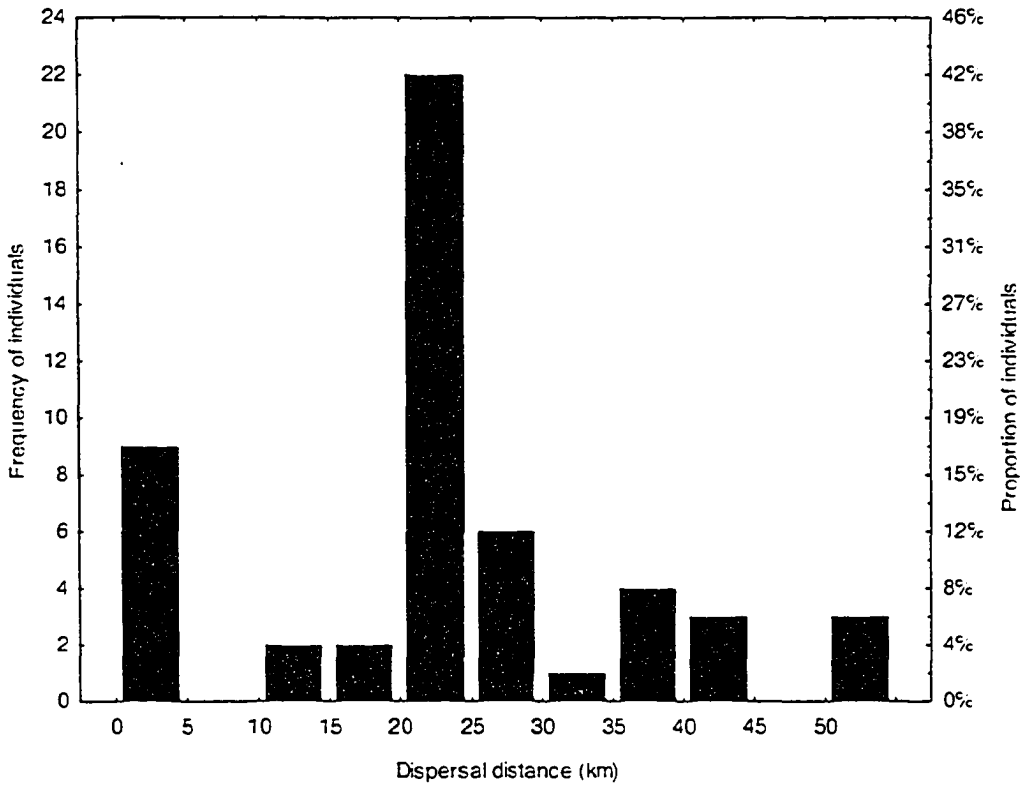


Figure 2.4: Frequency and proportion histogram of the estimated dispersal distance of individuals. Only those individuals from DFA of test dataset that had a *posterior probability* >90% are illustrated.



Chapter 3: Sagitta and lapillus: different microchemical recorders within the same fish.

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Abstract

Otolith microchemical studies have overwhelmingly favored the use of sagittal otoliths even though different otolith types (sagitta, lapillus, and astericus) likely vary in terms of their responses to environmental chemistry. Individuals of *Stegastes partitus* were collected from Turneffe Atoll, Belize (2002 and 2003) and Banco Chinchorro, Mexico (2003), from which sagittae and lapilli were removed for microchemical analysis. Wilcoxon matched pairs test, which compared elemental concentrations of sagitta and lapillus from the same fish, indicated significant differences in terms of Li, Mn, Rb, and Pb, such that Li, Mn, and Rb concentrations were greater in lapillus, while Pb concentrations were greater in sagitta. Therefore, lapillar and sagittal otoliths reflected the environment, but to varying degrees. To test which otolith types' chemistry was superior at discriminating populations, a linear discriminant function analysis (DFA) was conducted separately for sagittae and lapilli. Results indicated that although sagittal and lapillar otoliths discriminated populations with similar levels of confidence (average correct classification of 81%) they did so using a different suite of elements. Because of the elemental concentration differences among otolith types within the same fish, we asked whether combining lapillar and sagittal otolith chemistries would improve the discrimination of sampling locations/times. Results from this DFA indicated that correct classification increased to 94%, and therefore suggested that the use of elemental concentrations from both otolith types will improve discriminatory power, particularly in situations where differences among sites of interest are relatively small.

Introduction

The majority of teleosts have three types of otoliths (sagitta, lapillus, and astericus, listed in decreasing size) located within fluid (endolymph) filled sacs (sacculus, utriculus, and lagena in order of otolith types) of the inner ear system. These otoliths are used for sound reception and orientation in a three-dimensional environment (Campana 1999; Solner et al. 2003; Lombarte and Popper 2004). Prior to the 1960s, and as early as 1800s (Jones 2002), biologists using the unique properties of otoliths (i.e., their yearly growth banding pattern), determined the age of adult fish and hence the age structure of populations (Yoshihara 1955; Dannevig 1957; Hotta 1960). But it wasn't until the 1970s, that studies using otoliths became more prevalent in fisheries research. Panella's (1971) discovery of daily increments in otoliths, provided a means to investigate questions relating to juvenile life history, such as mortality, growth, and age structure (Figure 1.1). In addition to the use of otoliths as a record of time, the incorporation of trace elements from the environment onto the growing surface of the otolith (e.g., Sr- Farrell and Campana 1996; Gallahar and Kingsford 1996; Bath et al. 2000; Milton and Chenery 2001; Kennedy et al. 2002; Elsdon and Gillanders 2003; Ba- Bath et al. 2000; Milton and Chenery 2001; Kennedy et al. 2002; Elsdon and Gillanders 2003; Li- Milton and Chenery 2001; Pb- Geffen et al. 1998) led to the use of otoliths as recorders of environmental chemistry (Campana 1999).

Some of the earliest otolith microchemical work used the storage ability of otoliths to investigate issues of pollution (Macpherson and Manriquez 1977; Papadopoulou and Moraitopoulou-Kassimati 1977; and Papadopoulou et al. 1980) (Figure 1.1), while more recently, studies have successfully investigated the spatial arrangement of populations (e.g., Edmunds et al. 1989; Dove and Kingsford 1998;

Patterson et al. 1999; Swearer et al. 1999; Kingsford and Gillanders 2000; Hamer et al. 2003; Rooker et al. 2003) as well as the migration of individuals and resulting connectivity among populations (Milton et al. 1997; Campana et al. 2000; Kafemann et al. 2000; Milton et al. 2000; Thorrold et al. 1998 and 2001; Volk et al. 2000; Yamashita 2000; Secor et al. 2001; Forrester and Swearer 2002; Gillanders 2002; Chittaro et al. 2004).

Because the majority of otolith microchemical studies have used the sagitta, little information is available regarding the microchemical nature of lapillus. Both otolith types have been reported to develop prior to hatching, at which point they are approximately the same size (see Zhang and Rhuham 1989; Pisam et al. 2002; Wilson and Meekan 2002). Yet, because of differential growth rates with age (lapilli grow faster during early life, sagittae faster subsequently), the size of sagittal otoliths later surpasses that of lapillar otoliths (Campana and Gagne 1995). In terms of microchemistry, since growth rates (i.e., crystallization rate) vary between otolith types and the incorporation of trace elements varies with growth, it is likely that sagittal and lapillar otoliths reflect the environment differently (Campana and Gagne 1995). To our knowledge, the only known microchemical studies comparing sagittae and lapilli were for temperate (*Gadus morhua*, Atlantic cod, Campana and Gagne 1995) and subtropical species (*Mugil cephalus*, Flathead mullet, Meyer-Rochow et al. 1992; *Brevoortia patronus*, Gulf menhaden, Chesney et al. 1998). Of these studies, Campana and Gagne (1995) and Meyer-Rochow et al. (1992) showed significant differences between otolith types, but only the former study compared the ability of sagitta and lapillus to discriminate populations.

Here, we compare trace elemental concentrations of sagittal and lapillar otoliths from the same fish, *Stegastes partitus* (Bicolor damselfish), a common Caribbean reef fish that was collected at several sites over the course of two summers. The specific goals of this study were to determine a) if elemental concentrations vary between otolith types (sagitta and lapillus), b) which otolith type is superior at discriminating populations, and c) whether combining the elemental concentrations of otolith types results in improved population discrimination. Since both otolith types are found within similar sacs that are filled with endolymph containing environmentally derived trace elements, our null hypothesis is that the elemental concentrations are identical between lapillar and sagittal otoliths.

Materials and methods

A sample of individuals of *Stegastes partitus* (n=122) was selected from collections that were made along the Mesoamerican Barrier Reef System every other week from May to August in 2002 and 2003. The sample consisted of individuals that were collected from Turneffe Atoll, Belize, in 2002 (early June, 42 individuals, and early July, 38 individuals) and 2003 (late July, 26 individuals) and from Banco Chinchorro, Mexico, in 2003 (early June, 16 individuals) (Figure 3.1). Individuals ranged in size from 1.2-6.0 cm (standard length) with a mean of 2.3 cm (±1.0 cm standard error and median of 2.0 cm).

One sagitta and lapillus were removed per fish, mounted in Crystal Bond®, and polished in a transverse plane using 30-3 μm lapping film. In a class 100 clean room, multiple otolith sections (up to 35) were fixed to a microscope slide and cleaned by

placing them in a milliQ water bath to be sonicated for 2.5 minutes, triple rinsed with 95% ethanol, and then triple rinsed with milliQ water. Each slide was then allowed to dry overnight in a laminar flow HEPA-filtered fume hood.

Otoliths were chemically analyzed at the Great Lakes Institute of Environmental Research (University of Windsor), using laser ablation inductively coupled plasma mass spectrometry, LA-ICP-MS). A Thermo Elemental X7 ICP-MS was operated at low resolution with argon used as the carrier gas from the laser sampling cell. The laser sampling system is a purpose-built system (Fryer et al. 1995) based on a non-homogenized, high power, frequency-quadrupled (266 nm) Nd:YAG (neodymium-doped yttrium aluminum garnet) laser. Frequency was set at 20 Hz, flash lamp power was 1.15 kV, and a 1 mm pinhole beam constrictor was used to decrease the spatial resolution of the laser sampling. Based on these settings, laser ablation diameter and depth were approximately 15 and 45 μm, respectively. The laser beam was focused onto the sample using an Olympus® BX-51 petrographic microscope and an Optics For Research® 266 nm 10 X objective lens. The sampling system is more fully described in Crowe et al. (2003).

LA-ICP-MS data acquisition lasted 140 seconds with 60 seconds of instrument and gas background counts prior to the start of each ablation. The otolith portion of interest (i.e., transects at the otolith edge perpendicular to growth increments) was targeted using an automated microscope stage moving at a speed of approximately 5 μm/sec. A trace element-doped glass standard (National Institute of Standards and Technology, NIST, 610, a homogenous glass that is spiked with a range of elements of known concentrations) was analyzed in duplicate at the beginning and end of each

sample set to correct for instrument drift (20 samples). Calcium was used as an internal standard to compensate for signal variation caused by differences in mass of ablated material.

In total, 13 elements were analyzed by the LA-ICP-MS and chemical concentrations (parts per million) were calculated using Lamtrace software (van Achterbergh et al. 2001). The elemental signal selected for use in Lamtrace was limited to a 20 μm length at the edge of each sagitta and lapillus (in the sagitta this length corresponds to approximately 2-3 days based on estimates of settlement stage fish by Wilson and Meekan 2002). Although the growth rates of sagittal and lapillar otoliths differ, and our signal selection for lapillus likely accounts for a longer time period in the life of a fish relative to that for sagitta, we used the same length of ablation (transect length of 20 μm) for both otolith types in order to standardize for the amount of material ablated. Standardizing the amount of material ablated ensures that levels of detection do not vary between otolith types. In addition, we feel that any incorporation of additional lapillus material, relative to sagitta, will not cause concentration differences between otolith types since the site-attached nature of *S. partitus* means that this extra material will simply reflect the chemical concentration of the same environment. After signal selection, elements that met the following two criteria were included in statistical analyses: 1) concentrations of NIST samples were determined with a satisfactory precision (coefficient of variation less than 10%); and 2) concentrations in otoliths greater than detection limit for more than 50% of otoliths analyzed. For those elements that met the above criteria, outlier analysis was performed for each element such that any values

that were three times the interquartile distance were removed (see Fowler et al.1995; StatSoft 2001).

Since paired differences between sagitta and lapillus for Mg, Zn and Pb concentrations did not meet the assumption of normality for paired t-tests, a non parametric approach was used. Specifically, a Wilcoxon matched pairs test was used to investigate whether elemental concentrations differed between sagittal and lapillar otoliths from the same fish. A discriminant function analysis (DFA) was used to determine whether elemental concentrations from sagitta or lapillus were more useful at discriminating fish from different locations and times. Specifically, separate DFAs were conducted for each otolith type such that the grouping variable corresponded to four location/time sets (i.e., Tumeffe Atoll June 2002, Tumeffe Atoll July 2002, Tumeffe Atoll 2003, and Banco Chinchorro 2003). Additionally, a DFA was used to determine if there was any improvement in discriminatory power when trace elemental concentrations of both sagittal and lapillar otoliths were used in the same dataset (e.g., Sr concentrations for lapillus and sagittal otoliths were separate variables). Elements important in the DFA were indicated by factor correlations greater than $|0.33|$, which represents approximately 10% of the variance (Tabachnick and Fidell 1996). For each DFA, classification functions were produced that were used to indicate the percentage of individuals from each location/time that were correctly classified. Elements were $\log_{10}$ transformed to improve normality for the DFA.

Results and Discussion

Based on the two criteria (concentrations of NIST samples that had coefficients of variation less than 10% and concentrations in otoliths that were greater than detection

limit for more than 50% of otoliths analyzed), seven of 18 elements were retained for statistical analysis. Specifically, Li, Mg, Mn, Zn, Rb, Sr, and Pb were at concentrations sufficiently above detection limit to permit meaningful interpretations (Table 3.1). The Wilcoxon matched pairs test indicated significant differences between sagittal and lapillar otoliths in terms of Li, Mn, Rb and Pb (Figure 3.2). Campana and Gagne (1995) also reported significant differences in elemental concentrations between otolith types. They noted that these differences might be a result of the interaction between the method of analysis (solution of whole otoliths) and differential otolith growth rate with age. Specifically, differences between sagittal and lapillar otolith chemistry might arise in solution analyses if the fish experienced different habitats at different ages, and thus the lapillus, which grows faster during early development, would have more material from that time and location relative to the sagitta (see Campana and Gagne 1995). The influence of differential growth rates on chemistry was also highlighted by Meyer-Rochow et al. (1992) who compared the core chemistry of sagitta to whole (dissolved) lapillus, and found no differences in the concentrations of stable isotopes of carbon and oxygen. However, when whole sagitta were compared to whole lapillus, differences were detected. They suggested that the sagitta likely incorporated unique elemental concentrations later in life that did not occur at its core or in whole lapillus (or did not occur at sufficient concentrations in lapillus relative to the rest of the otolith). Because we targeted the otolith edge, we avoided any substantial influence of differential growth (that is incorporated in solution analysis) and thus were able to compare the chemical signals at a specific time in the fish's life.

The comparisons of elemental concentrations between otolith types indicated that concentrations of Li, Mn, and Rb were usually significantly greater in lapillar otoliths, while Pb was greater in sagittal otoliths (Figure 3.2). These substantial differences in elemental concentrations between sagittal and lapillar otoliths may indicate differential regulation of elements passing through their respective vestibules (i.e., sacculae and utriculus, respectively) and into the endolymph. Although this may help explain the relatively consistent pattern through time and space of greater Mn and Rb concentrations in lapillar otoliths and Pb concentrations in sagittal otoliths, it does not clarify why concentrations of Li (as well as Mg and Zn, and to a lesser degree Rb) increased in sagittae, but not in lapilli from 2002 to 2003 (which caused the change in concentration difference between sagittae and lapilli shown in Figure 3.2 and Table 3.1). Specifically, average Li, Mg, and Zn concentrations in sagittae increased 10, 3, and 3 fold, respectively, from 2002 to 2003, while their concentrations in lapilli increased 0, 1.5, and 0 fold, respectively (Table 3.1). Could the higher environmental concentrations in 2003 combined with differences in filtration of the saccular relative to the utricular vestibule result in the temporal variability observed for Li, Mg, and Zn in sagittae? In other words, could the environmental levels of Li, Mg, and Zn be relatively high in 2003, but because of ion regulation in the utricular vestibule they are not deposited onto the lapillus at concentrations similar to that of sagitta? Although higher environmental levels of Li, Mg, and Zn are likely in 2003 (since similar elemental concentrations were observed for sagittal otoliths from individuals collected throughout Tumeffe Atoll in 2003, see Chapter 2), it is unknown whether the saccular and utriculus vestibules would behave so differently under different environmental conditions.

The discrimination of individuals among their locations/times of collection was successful (81% of individuals were correctly classified) regardless of otolith type (Table 3.2). This result is similar to that of Campana and Gagne (1995), in which a relatively high degree of discrimination was also evident (94% and 83% for sagittal and lapillar otoliths, respectively). However, contrary to the findings of Campana and Gagne (1995), in which sagittal otolith chemistry indicated greater success in discriminating populations, we report no clear advantage for either otolith type, and thus suggest that either otolith type can be used to effectively discriminate populations.

By using the concentrations of all seven elements of both otolith types in a DFA, correct classification of individuals was improved to 94% (concentrations of 6 element/otolith type variables were used in the discrimination: $Li_{sagitta}$, $Mn_{sagitta}$, $Mg_{sagitta}$, $Mn_{lapillus}$, $Rb_{sagitta}$, and $Rb_{lapillus}$, in order of decreasing discriminatory importance). This improved discriminatory ability was shared between otolith types: the first discriminant root was best explained by elemental concentrations within sagittae (Li, Mg, and Mn) and lapilli (Mn), while elemental concentrations of sagittae and lapilli (Rb) were important in explaining the second discriminant root (Table 3.2; Figure 3.4). Consequently, this analysis highlights a potential increase in spatial and/or temporal discriminatory power when both otolith types were used.

This study indicated that there is considerable variability in elemental concentrations between otolith types, yet despite these differences, the ability to discriminate collections of individuals was similar for sagittal and lapillar otoliths. In addition, this study highlights that the combination of sagittal and lapillar otolith concentrations provides additional information that allows greater separation of fish

collected from different locations/times. Although more studies are needed to test the utility of using both otolith types (e.g., the cost associated with the analysis of both otolith types versus its benefits) we feel that the inclusion of both types will provide the improved level of chemical resolution necessary for studies at finer spatial and temporal scales, or in cases where chemical differences among sites of interest are relatively small.

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Table 3.1: Elements included in statistical analyses that were determined from the coefficient of variation (CV) of NIST samples being < 10% and > 50% of otolith samples with greater than detection limit. Average and standard deviation (SD) provided for detection limit by sample ablation, overall concentration, and concentrations per year and otolith type. Average CV is of 25 different sets of otolith ablations. All values are in parts per million.

Isotope measured	Average. CV (SD)	% of samples > detection limit	Average detection limit by sample (SD)	Overall average concentration (SD)	Average concentration 2002 Lapillus (SD)	Average concentration 2003 Lapillus (SD)	Average concentration 2002 Sagitta (SD)	Average concentration 2003 Sagitta (SD)
Sample size				101	68	33	68	33
Li (7)	6.42 (5.76)	97	0.06 (0.07)	1.20 (1.01)	1.50 (0.86)	1.75 (0.77)	0.21 (0.17)	2.09 (0.92)
Mg (24)	3.03 (2.48)	99	1.47 (1.46)	12.37 (9.58)	9.80 (7.61)	15.3 (11.1)	8.06 (4.74)	23.4 (9.37)
Mn (55)	4.60 (3.76)	80	0.29 (0.26)	1.03 (0.81)	0.89 (0.47)	2.18 (0.61)	0.30 (0.18)	1.66 (0.49)
Zn (66)	6.52 (5.36)	87	0.22 (1.30)	1.99 (1.95)	1.80 (1.86)	1.76 (1.20)	1.33 (1.52)	4.00 (2.27)
Rb (85)	7.56 (6.03)	81	0.04 (0.03)	0.08 (0.05)	0.12 (0.04)	0.09(0.06)	0.04 (0.04)	0.07 (0.03)
Sr (86)	1.67 (1.26)	100	0.53 (0.41)	3173 (795)	3121 (637)	3185 (526)	3281 (1098)	3042 (524)
Pb (208)	6.12 (4.16)	99	0.03 (0.02)	5.07 (8.90)	0.45 (0.78)	0.31 (0.24)	8.77 (10.96)	11.74 (10.1)

Table 3.2: Results of the discriminant function analyses (DFA) to differentiate groups (i.e., locations/times of fish collections) using elemental concentrations of sagitta, lapillus, and sagitta plus lapillus. Specifically, information is provided regarding Wilks' Lambda (the variance not explained by the discriminant functions), Chi-square (X^2) statistic (the measure of statistical significance) and the proportion of the variance represented by significant roots (%). Factor correlations >|0.3| are indicated for each element (Li, Mg, Mn, Zn, Rb, Sr, and Pb) and root. 'S' and 'L' denote concentrations from sagittal and lapillar otoliths, respectively.

DFA (ave. % correct)	Wilks' Lambda	Df	F	p<	X^2	df	p<	%	Root #	Li	Mg	Mn	Zn	Rb	Sr	Pb
Sagitta (81%)	0.05	21, 261	21.1	0.001	268	21	0.001	96	1	-0.69	-0.44	-0.66	-0.30			
									2			-0.37		-0.69		
Lapillus (81%)	0.15	21, 261	11.4	0.001	176	21	0.001	90	1			-0.63		0.72		
									2			0.50		0.56		
Sagitta & Lapillus (94%)	0.02	42, 249	16.5	0.001	358	21	0.001	93	1	0.60S	0.39S	0.58S 0.34L				
									2					-0.53S -0.39L		

Figure 3.1: Map of study locations (Turneffe Atoll, Belize and Banco Chinchorro, Mexico). Triangles and circles correspond to sampling that was conducted in 2002 and 2003, respectively.

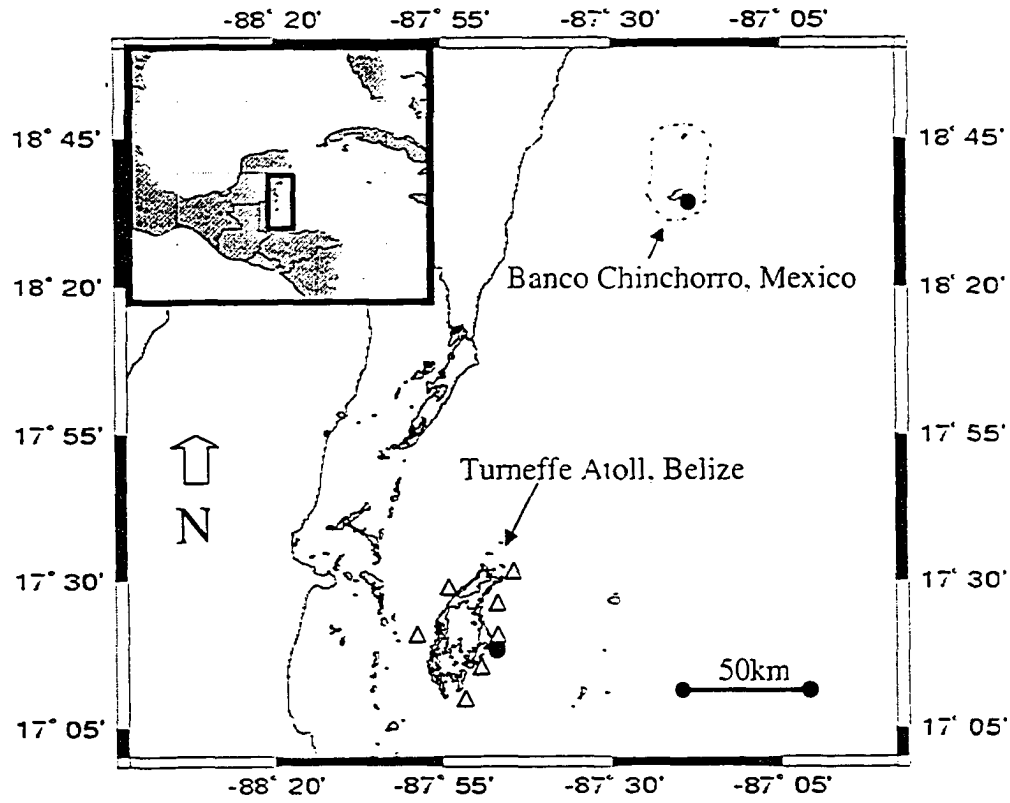


Figure 3.2: Differences in concentrations (ppm) between lapillar and sagittal otoliths for each fish (each bar represents one individual), in terms of Li, Mg, Mn, Zn, Rb, Sr, and Pb. Results are provided for the Wilcoxon matched pairs tests, which assessed whether there were significant differences in elemental concentrations between otolith types. BC and TA refer to Banco Chinchorro, Mexico and Turneffe Atoll, Belize, respectively.

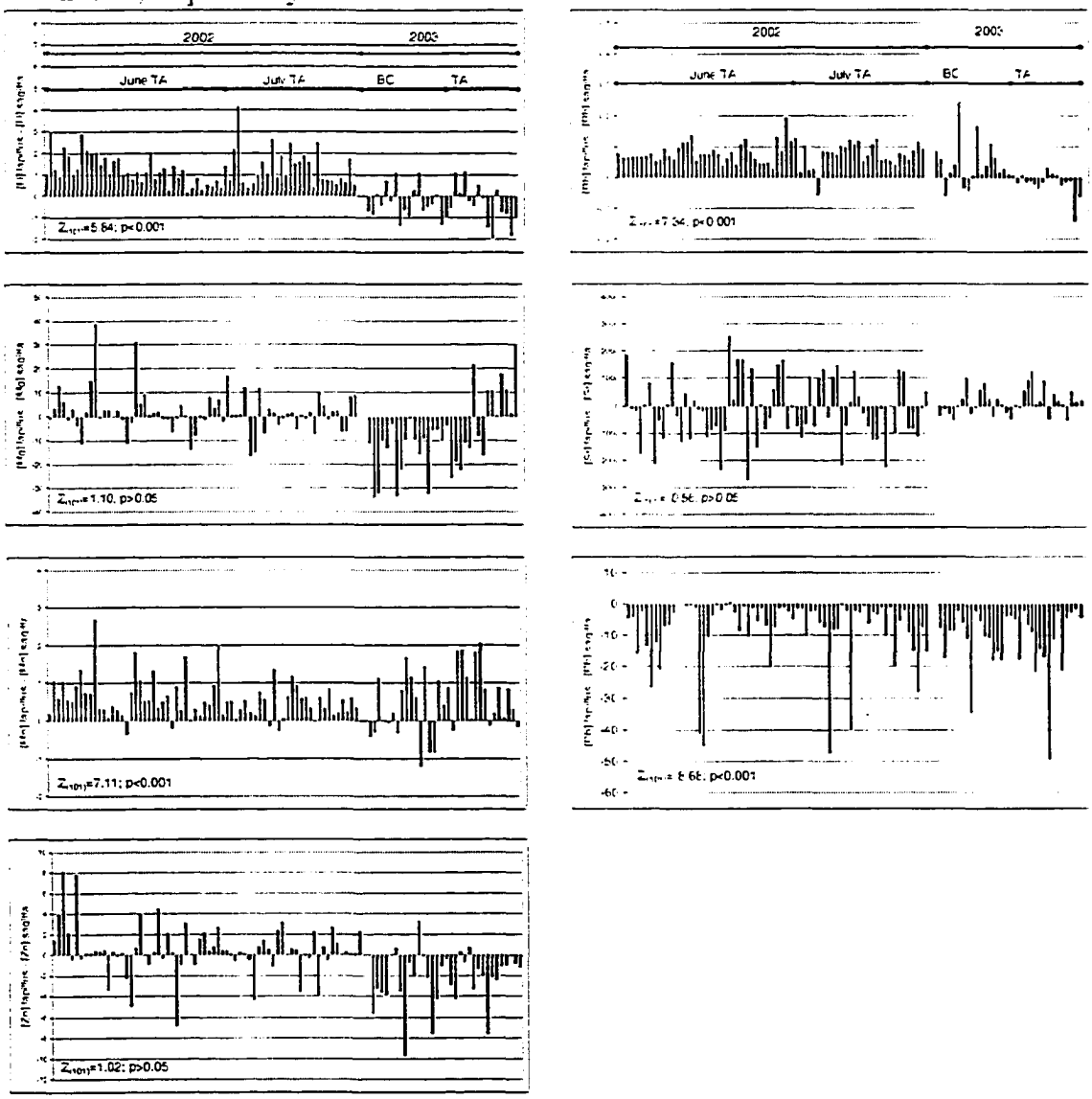
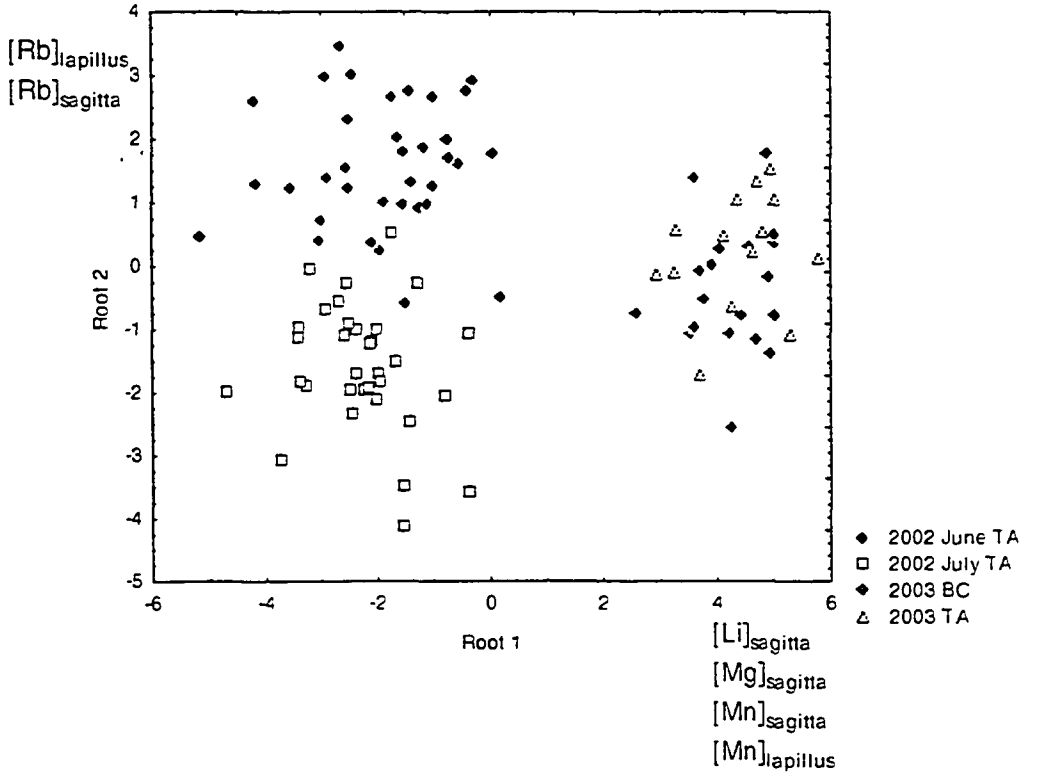


Figure 3.3: Plot of individuals from their locations/times of collection in DFA space using elemental concentrations from both otolith types. Elements are indicated that account for > 10% of the separation.



Chapter 4: Ontogenetic variability in the otolith microchemistry of *Stegastes partitus*.

A modified version of this chapter was submitted to Marine Biology with the following authors:

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Abstract

Otolith microchemistry can be used to assess pelagic larval fish connectivity by comparing spatially variable otolith edge chemistry (corresponding to the site of collection) to otolith core chemistry (corresponding to the site of hatching). However, because the otolith's edge and core represent different life stages, the deposition of trace elements may differ, thus complicating direct comparisons of edge and core chemistry to investigate connectivity. Here we present data from a field experiment designed to assess whether elemental concentrations of otoliths from *Stegastes partitus* vary ontogenetically. Specifically, elemental concentrations of embryo otoliths (3 days post-fertilization) and juvenile otoliths, which were collected at the same sites (Turneffe Atoll, Belize, and Banco Chinchorro, Mexico) and times (June and July 2003), were compared.

Separate MANOVAs for embryo otoliths and the edges of juvenile otoliths, which were used to investigate the microchemical variability between sites and times, revealed significant differences and thus an environmental influence to the otolith chemical signal. To assess whether otolith microchemistry varied with life history stage (i.e., showed ontogenetic variability) a nested MANOVA was carried out comparing otolith microchemistry of embryos and juvenile edges (nested within site/time). Results of this analysis indicated that trace elemental concentrations of embryo otoliths were significantly greater than those at the edges of juvenile otoliths. Because embryo trace elemental concentrations of Mn, Zn, Ba, Ce, and Pb were between 2 and 325 times greater than those of the corresponding juvenile otoliths, it was clear that the environment was not the primary control of otolith embryo microchemistry. Consequently, caution is warranted when interpreting environmental patterns of otolith cores, particularly when using them as a proxy for natal signatures.

Introduction

The development of new technologies (e.g., laser ablation IC-PMS) has enabled fish ecologists to re-evaluate issues of connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals; Mora and Sale 2002), in particular, the degree to which fish populations are open (consisting of populations connected primarily through the dispersal of individuals) or closed (populations not receiving a substantial number of immigrants) (see Swearer et al. 1999). The significance of understanding population connectivity is apparent when one considers that resource managers and conservationists make decisions regarding commercial and sport fisheries under the assumption that long distance dispersal of fish is frequent and thus populations are open (Mora and Sale 2002). For instance, the theoretical evidence for the use of marine protected areas as a fisheries management tool arose partially as a result of the view that populations are open and, therefore, through the reproduction by resident adults and the dispersal of their eggs and larvae, adjacent and distant fished areas are hypothesized to be seeded (see Russ 2002; Sale et al. 2005).

Attempts to quantify the connectivity of reef fish populations, have utilized artificial (e.g., tetracycline in otoliths, Jones et al. 1999) and natural (e.g., geochemical tags in otoliths, Swearer et al. 1999; gene frequencies, Taylor and Hellberg 2003) tags. Because most reef fish have a pelagic larval phase during which mortality is assumed high, any assessment of connectivity using artificial tags requires an exhaustive tagging and re-sampling process to detect movement. For example, after labelling the otoliths of approximately 10 million embryos of *Pomacentrus amboinensis* with tetracycline at a relatively isolated island (Lizard Island, Australia) Jones et al. (1999) observed that 15

out of 5000 individuals collected at settlement contained the tag. Subsequently, they estimated that 15 to 60% of the recruits were produced locally. Although this mark-recapture program was successful, the logistic difficulty associated with ensuring adequate marking and collection increases when areas that are more spatially complex (e.g., a network of islands, each with their own population) are investigated. Alternative approaches are needed.

Use of the natural microchemical signature of otoliths is an alternative technique that relies on the continual deposition of environmentally-derived trace elements onto the growing otolith surface, so that each otolith chemically records its surroundings (much like the electronic records of an airplane flight recorder). The successful use of natural otolith tags to understand connectivity (i.e., the degree to which populations are open or closed) requires that otolith chemistry reflect the environment (see evidence of this for Sr- Farrell and Campana 1996; Gallahar and Kingsford 1996; Bath et al. 2000; Milton and Chenery 2001; Kennedy et al. 2002; Elsdon and Gillanders 2003; Ba- Bath et al. 2000; Milton and Chenery 2001; Elsdon and Gillanders 2003; Li- Milton and Chenery 2001; Pb- Geffen et al. 1998) and that there is sufficient geographic variability in the environment to discriminate natal locations (Hamer et al. 2003). For example, to discern patterns of pelagic stage movement, Swearer et al. (1999) used *Thallassoma bifasciatum* (Bluehead wrasse) and the natural trace elemental differences between coastal and open ocean environments recorded in their otoliths to estimate that 35% of individuals were produced locally. In addition, Thorrold et al. (1998) first used elemental concentrations of trace elements and stable isotopes to classify juvenile *Cynoscion regalis* (Weakfish) to their natal estuary with a 60 to 70% success rate. Later, by examining adults spawned

during the initial collection, they identified that 60 to 81% of individuals that were analysed showed signs of site fidelity (i.e., adults returned to the estuary from which they were spawned) (Thorrold et al. 2001).

To assess pelagic larval connectivity, one approach is to compare spatially variable post-settlement otolith chemistry (i.e., which corresponds to the known site of collection) to otolith core chemistry (corresponding to the unknown site of hatching). Specifically, individuals would be collected throughout the study location and their otoliths chemically analysed at their edge. Since the edge of the otolith corresponds to material (e.g., trace elements) deposited just prior to collection, the chemistry of this region of the otolith corresponds to that from a known site (i.e., the site from which the individual was collected). Thus, the otolith edge chemistry of fish across all sites provides a time-specific chemical map of the study location. This chemical map can then be compared to the otolith core chemistry (which corresponds to the environmental chemistry at the natal location) of fish that were spawned during the initial collection. Any similarity in elemental concentrations between a site from the chemical map and the core chemistry of an individual likely indicates the hatching location of that fish.

However, an assumption of the above approach is that deposition of trace elements onto the growing otolith is consistent regardless of age (or physiological stage), and thus otolith chemistry from hatching individuals (i.e., core chemistry) should be equivalent to that of juveniles and adults (i.e., edge chemistry) from the same site and time. Work by Fowler et al. (1995) and de Pontual et al. (2003) on *Micropogonias undulatus* (Atlantic croaker) and *Solea solea* (Sole), respectively, highlighted that both ontogenetic and environmental influences were involved in determining the early stage

otolith chemistry. In addition, both Toole et al. (1993) and Otake et al. (1994) showed patterns in the concentration of Sr that appeared to be influenced by ontogenetic processes such as hatching and metamorphosis, in *Microstomus pacificus* (Dover sole) and *Anguilla japonica* (Japanese eel), respectively.

In this paper, we assess, using *in situ* experiments, whether otolith core and edge chemistry of a common Caribbean reef fish (*Stegastes partitus*) can be used to assess the movement of individuals. To our knowledge, this is the first study to investigate ontogenetic variability in otolith microchemistry using direct comparisons of otolith microchemistry of individuals at different life history stages (in particular in embryos and juveniles rather than through retrospecive analyses from adults). The specific aim of this study was to determine whether otolith microchemistry from *S. partitus* varies ontogenetically between embryos and juveniles, and if so, what patterns may be identified. Our null hypothesis was that the environment in which a fish resides primarily determines otolith microchemistry and therefore, no discrepancy is anticipated in terms of trace elemental composition between otoliths from embryos and juveniles.

Materials and methods

To assess ontogenetic variability in otolith microchemistry, this study used *Stegastes partitus*, Bicolor damselfish, a common Caribbean coral reef fish. *Stegastes partitus* (Class Actinopterygii, Order Perciformes, Family Pomacentridae) is a relatively small fish (maximum size approximately 10 cm) common on shallow coral reefs throughout the Caribbean, and are demersal spawners whose reproduction follows a lunar cycle (Knapp 1993). Specifically, females deposit a clutch of eggs (approximately 2000 eggs, Cole and Sadovy, 1995) on the underside of coral, rock, or rubble in a male-

defended territory, approximately 3 to 5 days following a full moon (Robertson et al. 1988: Knapp 1993). At sunset, 3.5 days after egg deposition, embryos hatch and begin their 29 to 35 day pelagic existence (Robertson et al. 1988: Wellington amd Victor 1989: Wilson and Meekan 2002). Following settlement, individuals are relatively sedentary, occupying a small territory for the remainder of their lives (Robertson et al. 1988).

Stegastes partitus was chosen for the study because it is very abundant and relatively easy to catch with hand nets and clove oil. Furthermore, various aspects of their biology make them an ideal species to use in addressing issues of ontogenetic variability and connectivity. For instance, because of a relatively sedentary post-settlement existence, their otolith chemistry will be influenced by an exposure history exclusive to a specific area, without any confounding effect that could be related to movement if a larger, more mobile species was examined. In addition, since *S. partitus* readily occupy artificial substrate and are demersal spawners, it is possible to more easily collect and manipulate embryos compared to that of pelagic spawners.

To investigate ontogenetic variability in otolith microchemistry of *Stegastes partitus*, field experiments were conducted at Banco Chinchorro, Mexico, starting June 9 and July 20, and at Turneffe Atoll, Belize, starting July 20, 2003 (Figure 4.1). For simplicity, these three location-time combinations are referred to throughout the text as location/time. An experiment consisted of a haphazard placement of 30 terra cotta roofing tiles (approximately 50 cm x 30 cm x 10 cm) in back reef sections (approximately 4 m depth) at both locations, which were monitored daily for the deposition of *S. partitus* eggs. Monitoring of tiles commenced 3 days prior to the full moon and lasted until the next new moon. Embryos were collected prior to or at hatching

(i.e., 3.5 days following deposition, Knapp, 1985) by scraping them into a large Ziploc® bag. Collected embryos were then transferred to a 20 mL vial (each vial corresponded to the tile from which embryos were collected for a given time, but could consist of eggs from multiple females) containing 95% ethanol. Immediately following the collection of embryos, a minimum of 5 juveniles (average standard length of 2.6 cm and standard deviation of 1.5 cm), and a minimum of two 30 mL water samples, were taken from the vicinity. Water samples were treated with 0.15 mL of ultrapure nitric acid to prevent biological growth, and stored at ambient temperatures.

Sagittae from juveniles were mounted in Crystal Bond® and polished in a transverse plane using 30-3 μm lapping film. In a class 100 clean room, multiple otolith sections (up to 35) were fixed to a microscope slide and cleaned by placing them first in a milliQ water bath that was sonicated for 2.5 minutes, followed by triple rinsing with 95% ethanol, and triple rinsed in milliQ water. Each slide was then allowed to dry overnight in a laminar flow, HEPA-filtered fume hood.

Otoliths of embryos were extracted using a novel technique. Using acid-washed pipettes, embryos contained within 1mL of ethanol (in which they were stored) were transferred to a 10 mL acid-washed Pyrex® vial. Using a new acid-washed pipette, approximately 8 mL of NaOCl (household bleach) was added to the vial containing embryos in order to dissolve the embryo tissue. The vial was sealed and agitated for 5 minutes. With a new acid-washed pipette, the entrained material (i.e., otoliths) was transferred to the center of 0.45 µm pore size filter paper (Teflon® PTFE membranes) placed atop of a Nalgene® reusable polysulfone filter holder and receiver (250 mL capacity), which was connected to a vacuum pump. After 1 minute of filtration of the

bleach solution containing entrained otoliths, milliQ water was added (three rinses of 2 mL each), to ensure that any excess bleach residue was removed from the otoliths. Otoliths were removed from the filter paper using double-sided tape that was subsequently fixed to a microscope slide. Each slide was then allowed to dry overnight in a laminar flow, HEPA-filtered fume hood.

Otoliths and water samples were analyzed in the ultratrace metals laboratory at the Great Lakes Institute for Environmental Research (University of Windsor). Otoliths were analyzed using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and water samples were analyzed using solution-based ICP-MS.

The purpose-built laser ablation system comprises a Continuum Surelite I non-homogenized, high power (2 mJ/pulse), frequency-quadrupled (266 nm) solid state neodymium-doped yttrium aluminum garnet (Nd:YAG) pulsed (20 Hz) laser. Laser ablation was conducted within an Ar-filled sample cell mounted to an Olympus BX-51 polarizing light microscope. The microscope was fitted with a specially-constructed Optics for Research 10X objective lens and a computer-controlled, motorized stage for precise, programmable X-Y-Z control. The laser pulse energy was reduced by lowering the flashlamp power to 1.15 kV (approximately 50% power) and the incident laser beam diameter was reduced from 5 mm to 1 mm diameter using a pinhole. The combination of reduced laser power and beam diameter and the 10 X microscope objective lens resulted in an ablation spot with a diameter of approximately 15 μm and a depth of approximately 45 μm.

Elemental analysis of otolith and water samples was conducted using a ThermoElemental X7 quadrupole ICP-MS. For laser ablation analyses, the spray

chamber and nebulizer were removed and the Ar gas stream containing the ablated sample was introduced directly into the ICP-MS torch. Each laser analysis consisted of a total of 140 seconds of data acquisition comprising an initial 60 seconds of preablation combined instrument and gas backgrounds and up to 80 seconds of sample ablation. For juvenile otoliths, a transect of the portion of the otolith of interest in this study (i.e., 20 µm at the otolith edge, perpendicular to growth increments) was analyzed at a rate of approximately 5µm/sec. This corresponds to the approximately 2 to 3 days in the life of the fish immediately prior to capture based on estimates of settlement stage fish by Wilson and Meekan (2002). For embryo otoliths, the entire otolith (mean diameter of 18 µm ± 3.2 µm), analyzed at a rate of approximately 5 µm/sec. was used. Although growth rates of juvenile and embryo otoliths differ, analyses were standardized to 20 µm to ensure comparable detection limits were achieved between the juvenile and embryo otolith analyses. Each laser ablation experiment typically consisted of 20 samples and a trace element-doped glass standard (National Institute for Standards and Testing, NIST 610, a homogenous glass that is spiked with a range of elements of known concentrations) that was analyzed in duplicate at the beginning and end of each experiment, in order to evaluate and correct for instrument drift. Each otolith was analyzed for 13 elements (see Table 4.1 for those elements used in statistical analyses).

For solution analyses, the ICP-MS spray chamber was fitted with a Glass Expansion Sea Spray nebulizer, which is better suited to the analysis of water samples containing high dissolved solids (e.g., ocean water). Prior to analysis, each water sample was diluted by a factor of 100 times using a 1% ultra pure nitric acid and milliQ water solution containing known concentrations of three elements (beryllium, indium and

thallium). All calibration standards and blanks were also diluted, using the same internal standard-spiked solution, so that instrument mass bias and drift and sample matrix effects could be identified, quantified and corrected for. The water samples were naturally aspirated (i.e., not pumped) by the nebulizer into the spray chamber, which improved instrument stability. We analysed each water sample for 29 elements (see Table 4.1 for those elements used in statistical analyses).

For laser ablation analyses, the stoichiometric abundance of Ca in ideal $CaCO_3$ was used as an internal standard to calculate concentrations of all the other elements in the otoliths and to compensate for variability in the volume of ablated material (i.e., ablation yield). Quantitative elemental abundances were calculated using LAMTRACE software (van Achterbergh et al. 2000). For solution analyses, quantitative elemental abundances were calculated using custom spreadsheet software designed to correct for instrument and sample matrix biases.

Quantitative concentration data that met the following two criteria were included in statistical analyses: 1) concentrations of NIST samples were determined with a satisfactory precision (coefficient of variation less than 10%); and 2) concentrations in otoliths were greater than the detection limit for more than 50% of otoliths analyzed. Outlier analysis was conducted such that any value that was greater than three times the interquartile distance was rejected (see Fowler et al.1995; StatSoft 2001). All data were $\log_{10}$ transformed to improve normality.

Separate multivariate analysis of variance (MANOVA) were used to test for differences in embryo and juvenile otolith elemental concentrations among the three locations/times (i.e., June and July for Mexico and July for Turneffe). Dependent

variables were concentrations of trace elements and the factors of the design were locations/times. A significant MANOVA was followed with direct discriminant function analysis (DFA), to determine which elements best differentiate individuals to the locations/times from which they were collected. To assess ontogenetic variability in otolith microchemistry, a nested MANOVA was used to compare otolith chemistry of embryos and juveniles. Dependent variables were concentrations of trace elements and the factors of the design were life stage (i.e., embryo or juvenile) nested within locations/times.

Results

Based on the two criteria (concentrations of NIST samples having coefficients of variation < 10%, and concentrations in otoliths being greater than the detection limit for > 50% of otoliths analyzed), several elements were retained for statistical analysis. Specifically, Mn, Zn, Sr, Sn, Ba, Ce, and Pb were at concentrations sufficiently above detection limits to permit meaningful interpretation (Table 4.1). Outlier analysis removed 6 juveniles (2 from Mexico/June and 4 from Belize/July) from 51 collected individuals (25 from Mexico/June, 5 from Mexico/July, and 21 from Belize/July), while outlier analysis removed 41 embryos (14 from Mexico/June, 19 from Mexico/July, and 8 from Belize/July) from 242 (114 from Mexico/June, 85 from Mexico/July, and 43 from Belize/July) that were collected.

Although assumptions of homogeneity of variance were violated for Zn, Sr, and Sn, similar results were obtained for both parametric and non-parametric analyses and, therefore, only parametric tests are reported. The MANOVA comparing elemental composition of embryo otoliths, indicated significant differences among locations/times

(Wilks' Lambda=0.18; df=14,382; F=36.7; p<0.001). Subsequent univariate analyses indicated that concentrations of Mn, Zn, Ba, and Ce differed significantly among locations/times (Table 4.2a). DFA comparing variability in elemental concentrations of embryo otoliths from the three locations/times indicated significant discrimination that produced two significant functions (chi-squared statistic = 331.1, df=14, p< 0.001) explaining 93% of the variation. The partial Wilks' Lambda was lowest for Zn and Pb (0.37 and 0.73, respectively) suggesting that these elements contributed relatively more to the discrimination of embryos among locations/times, compared to Mn, Sr, Sn Ba, and Ce (0.93, 0.98, 0.99, 0.91, and 0.98, respectively). Based on DFA factor correlations greater than |0.3| (i.e., accounting for approximately 10% of the variation, Tabachnick and Fiddel 1996), root 1 was best explained by Zn, while Ba and Ce explained root two (Figure 4.2a). Overall, the DFA indicated that otolith microchemistry varied sufficiently among locations/times to permit the correct classification of 97%, 92%, and 57% of the embryos to Mexico/June, Mexico/July, and Belize/July, respectively.

The MANOVA comparing the elemental composition of juvenile otoliths, indicated significant differences among locations/times (Wilks' Lambda=0.48; df=14,60; F=1.9; p<0.05). Subsequent univariate analyses indicated that concentrations of Zn and Sr differed significantly among locations/times (Table 4.2b). DFA comparing variability in elemental concentrations of juvenile otoliths among locations/times, indicated significant discrimination that produced one significant function (chi-squared statistic = 24.1, df=14, p< 0.05) explaining 64% of the variation. The partial Wilks' Lambda was the lowest for Zn and Sr (0.78 and 0.79, respectively) suggesting that these elements contributed relatively more to the discrimination of embryos among locations/times,

compared to Mn, Sn, Ba, Ce, and Pb (0.88, 0.96, 0.99, 0.91, and 0.96, respectively). Based on DFA factor correlations greater than |0.3|, root 1 was best explained by Zn, Sr, and Pb (Figure 4.2a). Overall, the DFA indicated that otolith microchemistry varied sufficiently among sampling locations/times to permit the correct classification of 77%, 40%, and 77% of the juveniles to Mexico/June, Mexico/July, and Belize/July, respectively.

The nested MANOVA used to assess ontogenetic variability in otolith elemental concentrations between embryos and juveniles, indicated significant differences among locations/times (Wilks' Lambda=0.68; df=14,454; F=6.82; $p<0.001$) as well as between the otoliths of embryos and juveniles nested within locations/times (Wilks' Lambda=0.05; df=21,652; F=52; $p<0.001$). Univariate analyses indicated significant differences in trace elemental concentrations among sampling locations/times in terms of Mn, Zn, Ba, and Ce (Table 4.2). In addition, univariate analyses indicated significant differences in trace elemental concentrations between embryo and juvenile otoliths nested within locations/times in terms of Mn, Zn, Sn, Ba, Ce, and Pb, such that concentrations of these elements in embryos were between 2 to 325 times greater than that in juveniles and 2 to 94 times greater than that of water samples (Table 4.1). Interestingly, concentrations of Sr did not differ significantly between juvenile and embryo otoliths, and the ratio of Sr concentrations in embryo otoliths to that of water were close to one for each of the three location/times (Table 4.1).

Discussion

Using otolith microchemistry to determine the natal origin of fish requires chemical information from the corresponding time in a fish's life (i.e., hatching) and an

ability to link it to the related location. This link can be formed by matching the elemental concentrations of the natal portion of the otolith (which corresponds to the core of the otolith) to the post-settlement portion of the otolith (which corresponds to the edge of the otolith, and therefore the site from which the fish was collected). Consequently, the success of otolith microchemical studies to investigate the pelagic movement of larvae depends upon the degree to which the otoliths elemental composition reflects the environment in which the fish resides, and whether this is consistent regardless of life history stage. Here, we conducted an *in situ* experiment to assess ontogenetic variability in otolith microchemistry, in which the otoliths of embryos were microchemically analyzed and compared to that of juveniles from the same location and time. To our knowledge, this is the first investigation of ontogenetic variability to use the otolith microchemistry of embryos, instead of retrospective analyses of the cores of older fish (see Fowler et al. 1995; de Pontual et al. 2003). Accordingly our chemical analyses of embryo otoliths represent genuine natal signatures, while retrospective analyses could (unintentionally) incorporate material peripheral to the core (because of difficulty in identifying and targeting the core microstructure), thus confounding the results of the particular analysis. For instance, Jones and Chen (2003) reported that retrospective analyses of the core region of juvenile otoliths would likely encompass a region larger than what was intended. Specifically, they reported that a maximum crater depth of 80 μm was sampled even though the laser diameter was 10μm together with a relatively low laser pulse frequency (5 Hz) and laser power (25%). Consequently, otolith material would be analyzed from a time in the life of the fish that was not necessarily of interest to the researcher. Based on these estimates and our instrument settings, if we had used

retrospective analyses of juvenile otoliths it would be unlikely to confine our microchemical sampling to the natal signature, which for *S. parvus* embryos was approximately a 20 and 15 μm diameter and depth (pers. obs. P.Chittaro), respectively. Therefore, the use of embryo otoliths meant that only material of interest was analyzed.

During embryonic development and otolith formation, if otolith elemental concentrations were entirely under physiological control, then variability in environmental concentrations would have no effect on the composition of embryo otoliths. However, our analyses of the elemental signatures of embryo otoliths indicated significant variability that permitted the accurate classification of embryos to their specific locations/times (Table 4.2a and Figure 4.2a). Similarly, juvenile edge chemistry varied significantly among locations/times but indicated a greater degree of overlap in otolith microchemistry than did embryo otoliths, and thus a reduced level of discrimination (i.e., juveniles edge chemistry indicated an average correct classification of 72% compared to 89% for embryos) (Table 4.2b and Figure 4.2b). In addition, there were differences in the elements that varied significantly among locations/times for juvenile and embryo otoliths. Consequently, both juveniles and embryo otolith chemistry appeared to reflect the environment in which they resided, but to varying degrees.

Despite the likely environmentally influenced chemical variability in otolith chemistry, the concentrations of trace elements between life history stages (embryos and juveniles) were significantly different (Table 4.2c), such that we rejected our null hypothesis of a lack of ontogenetic influence in otolith microchemistry. Similarly, Fowler et al. (1995) and de Pontual et al. (2003) reported significant ontogenetic variability in otolith microchemistry of *Micropogonias undulatus* (Atlantic croaker) and

Solea solea (Sole), respectively. However, the incongruity between this study and that of Fowler et al. (1995) and de Pontual et al. (2003) is that concentrations for each trace element in larval otoliths were between 2 to 235 times greater than that from juvenile edge chemistry, and 2 to 94 times greater than water samples (Table 4.1). The question thus becomes, what was causing the elevated concentrations in embryo otoliths relative to the chemistry of juvenile otoliths and water samples?

To our knowledge, only Michibata and Hori (1979) and Brophy et al. (2004) reported such elevated concentrations (but for Mn only). Michibata and Hori (1979) observed these elevated Mn levels in eggs and embryos of the freshwater fish *Oryzias latipes* (Japanese rice fish), while Brophy et al. (2004) observed it in retrospective analyses of juvenile *Clupea harengus* (Atlantic herring) and *Sprattus sprattus* (Sprat). Brophy et al. (2004) determined that the elevated concentrations were not the result of water or sediment contributions, but instead speculated that it might be linked to aspects of variation in otolith crystal structure and/or embryonic development.

The influence of changes in otolith crystal structure, such as vaterite and calcite, compared to the more common aragonite, are not well understood, but has been linked to variation in the deposition of trace elements (de Pontual and Geffen 2002). For instance, depleted Sr concentrations were observed to correspond with areas of vaterite for regions of otoliths of *Stenodus leucichthys* (Inconnu) and *Oncorhynchus tshawytscha* (Chinook salmon) (Brown and Severin 1999 and Gauldie 1996, respectively). Alternatively, calcite in the shell of albalone has been shown to have higher concentrations of some elements relative to aragonite portions (Bettiol et al. 1999) and, since calcite portions have been observed within otoliths (see work by Strong et al. 1986, on *Pollachius virens*, Pollock),

it is possible that elevated concentrations may occur in calcite regions of otoliths (Brophy et al. 2004). Although, crystal polymorphism can influence elemental concentrations, it is too early to suggest its relative importance in explaining our patterns. Clearly, more work is required regarding calcium carbonate polymorphs and their influence on the deposition of trace elements.

With regards to embryonic development and its role in elevated elemental concentrations, Volk et al. (2000) examined the maternal effects on otolith chemistry by crossing adult salmon that were captive within freshwater systems their entire life, to those that were wild anadromous. Results of this study indicated that where the mother lived and not necessarily the environment the eggs were spawned influenced the concentrations of Sr in the offspring. In fact, from the time salmon eggs are spawned until hatching, Volk et al. (2000), regarded them as a closed system in which the source of trace elements in the otolith was from the yolk sac. Thus, influences from the maternally associated environment (in this case freshwater or saltwater salmon habitat) are likely to have been transferred to the offspring via its yolk sac. In *Stegastes partitus*, embryonic and early larval development is maintained by yolk sac nutrients that are depleted two days after hatching (Wilson and Meekan 2002), and thus these stores could help explain the observed elevated elemental concentrations in embryo otoliths.

Since embryos were collected hours prior to hatching it is likely that the yolk sac is distorting any environmental signal in their otoliths. The relative yolk sac contribution could also explain the smaller signal duration and lower concentration of Mn observed in *Sprattus sprattus* (Sprat) compared to *Clupea harengus* (Atlantic herring), reported by Brophy et al. (2004). Specifically, *S. sprattus* and *C. harengus* have a yolk sac that is

absorbed at approximately 16 and 5 days post-hatch, respectively, which corresponds to an otolith diameter of approximately 21 µm for *S. sprattus* (Dulcic 1998) and 30 to 36µm for *C. harengus* (based on Figures 2, 3 and 4 in Fox et al. 2003). Based on both species otolith diameter at yolk sac absorption, and the 40µm laser ablation diameter used by Brophy et al. (2004), it is likely that the higher Mn concentrations observed for *C. harengus* resulted from the incorporation of material derived from the yolk sac, while the lower Mn concentrations observed for *S. sprattus* resulted from a proportionally higher incorporation of otolith material derived from the environment. In other words, when targeting the core of *S. sprattus* otoliths, a 40 µm ablation diameter would have removed a greater amount of material outside of the core, and thus material that was deposited after yolk sac absorption, compared to that of *C. harengus*, in which almost the entire ablation is targeting only yolk sac-derived material. In addition, difficulties in targeting the core of *C. harengus*, and thus a likely incorporation of peripheral material, may have resulted in their inability to detect Mn concentrations in 14% of their 97 individuals.

The elevated elemental concentrations observed in embryo otoliths may also have resulted from Ca-binding proteins in the blood (Kalish 1989 and 1991) and their variation with age or ontogeny. According to Kalish (1989 and 1991), there is a positive correlation between Ca in the blood and that in the endolymph (i.e., the fluid surrounding each otolith), yet because of Ca-binding proteins in the blood, there is a decrease in free Ca that is available to enter the endolymph. Therefore, if Ba concentrations (for example) remained constant in the blood but free Ca decreased, the result would be a relative increase in Ba levels in the endolymph and concomitant increase in Ba in the otolith (Kalish 1989 and 1991). Since Kalish (1989) reported that the number and type of

proteins in the blood influenced age-related variation in otolith Sr concentration, it is possible that during embryonic development the proportion of Ca-binding proteins limits the amount of Ca entering the endolymph and thus the otolith, relative to that during the juvenile life stage.

Finally, a large proportion of protein relative to calcium carbonate (in the form of aragonite) in the embryo otolith of *Stegastes partitus* (3 days post-fertilization) may cause the observed elevated concentrations. For example, it was not until 50 h post-fertilization (corresponding to a 16 μm diameter otolith) that Ca was first detected in the edge of *Danio rerioi* (Zebrafish) otoliths (corresponding to a 1 μm width) (Pisam et al. 2002). Consequently, microchemical analyses of 3-day post fertilization embryos would consist largely of a protein nucleus with an exterior portion containing Ca. Two problems arise from this possibility. First, since little is known about the trace elemental composition of otolith protein portions, it is possible that our elemental concentrations are related to the protein nucleus containing high concentrations of trace elements (possibly derived from the yolk sac). Secondly, in the data acquisition process, Ca is used as the internal standard since it comprises 90-95% of the otolith (see Pisam et al. 2002). However, in embryonic otoliths, the concentration of Ca may be less significant, and thus using it as an internal standard may lead to inaccurate estimates of trace elemental concentrations.

In conclusion, we have shown that the embryonic otolith chemical signal is different from that of juveniles and water collected at the same location and time, and thus rejected our null hypothesis of a lack of ontogenetic variability in otolith microchemistry. Consequently, analyses of connectivity that are based on trace elemental concentrations of otolith cores and edges should be interpreted cautiously. We

have speculated that crystal structure (i.e., aragonite versus vaterite or calcite) and/or embryonic development (i.e., yolk sac contribution, Ca-binding proteins, and otolith protein content) influenced the elevated concentrations in embryo otoliths relative to that of juveniles. Clearly, in order to make meaningful ecological interpretations of the natal elemental concentrations of otoliths, more information is required regarding the crystal structure and embryonic development, and how it influences elemental deposition in otoliths.

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Table 4.1: Average concentration (standard deviation, SD) of elements per sample (embryo otolith, juvenile otolith or water) per location/time. Ratios of concentrations of embryo otoliths: water samples and embryo otoliths:juvenile otoliths are provided along with average detection limit for embryo and juvenile otoliths, average coefficient of variation (CV) per element and percentage of samples greater than detection limit. Average CV is based on 25 different sets of otolith ablations.

Location/time	Sample	Average concentration (SD) (standardized to calcium)							
		N	Mn55 Manganese	Zn66 Zinc	Sr86 Strontium	Sn120 Tin	Ba138 Barium	Ce140 Cerium	Pb208 Lead
Mexico/June	Embryo otoliths	114	$1.7x10^{-01}$ ($1.6x10^{-01}$)	$1.1x10^{-01}$ ($1.2x10^{-01}$)	$9.1x10^{-03}$ ($7.2x10^{-03}$)	$1.9x10^{-05}$ ($2.0x10^{-05}$)	$1.6x10^{-01}$ ($9.1x10^{-01}$)	$1.9x10^{-05}$ ($1.1x10^{-05}$)	$5.6x10^{-05}$ ($3.6x10^{-05}$)
	Juvenile otoliths	25	$4.1x10^{-06}$ ($1.5x10^{-06}$)	$1.3x10^{-05}$ ($8.3x10^{-06}$)	$7.0x10^{-03}$ ($8.9x10^{-01}$)	$4.5x10^{-07}$ ($5.4x10^{-07}$)	$1.6x10^{-05}$ ($7.2x10^{-06}$)	$3.2x10^{-07}$ ($3.7x10^{-07}$)	$3.0x10^{-05}$ ($2.5x10^{-05}$)
	Water	2	$6.5x10^{-01}$ ($9.0x10^{-01}$)	$7.9x10^{-05}$ ($9.9x10^{-05}$)	$1.9x10^{-02}$ ($8.4x10^{-05}$)		$1.7x10^{-05}$ ($3.7x10^{-06}$)	$4.2x10^{-07}$ ($4.7x10^{-07}$)	$3.3x10^{-05}$ ($3.6x10^{-05}$)
	Embryo/Water		1	1.4	1		94	45	1.7
	Embryo/Juvenile		41	8	1	42	100	59	1.9
Mexico/July	Embryo otoliths	85	$3.0x10^{-01}$ ($2.0x10^{-01}$)	$3.9x10^{-01}$ ($4.2x10^{-01}$)	$7.0x10^{-03}$ ($3.9x10^{-03}$)	$1.6x10^{-05}$ ($1.0x10^{-05}$)	$9.3x10^{-01}$ ($7.7x10^{-01}$)	$1.2x10^{-05}$ ($9.2x10^{-06}$)	$4.5x10^{-05}$ ($3.3x10^{-05}$)
	Juvenile otoliths	5	$5.2x10^{-06}$ ($5.7x10^{-07}$)	$1.2x10^{-05}$ ($7.9x10^{-06}$)	$8.8x10^{-03}$ ($1.8x10^{-01}$)	$3.4x10^{-07}$ ($6.5x10^{-08}$)	$2.9x10^{-05}$ ($2.6x10^{-05}$)	$5.3x10^{-07}$ ($6.4x10^{-07}$)	$2.4x10^{-05}$ ($1.7x10^{-05}$)
	Water	4	$2.8x10^{-05}$ ($1.0x10^{-05}$)	$8.4x10^{-05}$ ($2.6x10^{-05}$)	$1.9x10^{-02}$ ($1.6x10^{-03}$)		$1.8x10^{-05}$ ($1.5x10^{-06}$)	$4.9x10^{-07}$ ($3.4x10^{-07}$)	$1.8x10^{-05}$ ($5.8x10^{-06}$)
	Embryo/Water		10	47	1		53	25	2.5
	Embryo/Juvenile		58	325	1	47	32	23	1.9
Belize/July	Embryo otoliths	43	$2.6x10^{-01}$ ($2.1x10^{-01}$)	$1.5x10^{-01}$ ($2.1x10^{-01}$)	$8.8x10^{-03}$ ($4.3x10^{-03}$)	$1.9x10^{-05}$ ($2.3x10^{-05}$)	$6.4x10^{-01}$ ($5.9x10^{-01}$)	$1.1x10^{-05}$ ($9.4x10^{-06}$)	$5.3x10^{-05}$ ($4.1x10^{-05}$)
	Juvenile otoliths	21	$3.8x10^{-06}$ ($3.7x10^{-07}$)	$6.6x10^{-06}$ ($3.6x10^{-06}$)	$7.9x10^{-03}$ ($1.4x10^{-01}$)	$3.9x10^{-07}$ ($1.5x10^{-07}$)	$2.1x10^{-05}$ ($1.1x10^{-05}$)	$2.6x10^{-07}$ ($5.3x10^{-07}$)	$2.0x10^{-05}$ ($1.5x10^{-05}$)
	Water	6	$2.6x10^{-05}$ ($2.9x10^{-05}$)	$8.4x10^{-05}$ ($8.9x10^{-05}$)	$1.8x10^{-02}$ ($1.2x10^{-03}$)		$1.7x10^{-05}$ ($3.6x10^{-06}$)	$3.3x10^{-07}$ ($1.7x10^{-07}$)	$1.3x10^{-05}$ ($4.6x10^{-06}$)
	Embryo/Water		10	18	0.5		37	32	4
	Embryo/Juvenile		68	227	1	49	30	42	2.6
Average detection limit for embryos (SD)			$7.7x10^{-05}$ ($3.4x10^{-01}$)	$6.8x10^{-01}$ ($8.9x10^{-01}$)	$2.5x10^{-01}$ ($1.8x10^{-03}$)	$1.4x10^{-05}$ ($5.0x10^{-05}$)	$1.1x10^{-01}$ ($8.2x10^{-01}$)	$3.2x10^{-06}$ ($1.7x10^{-05}$)	$6.5x10^{-06}$ ($5.0x10^{-05}$)
Average detection limit for juveniles (SD)			$1.7x10^{-06}$ ($4.6x10^{-07}$)	$4.2x10^{-07}$ ($1.0x10^{-07}$)	$1.2x10^{-06}$ ($3.2x10^{-07}$)	$1.3x10^{-07}$ ($3.3x10^{-08}$)	$8.8x10^{-06}$ ($2.6x10^{-06}$)	$4.9x10^{-07}$ ($2.6x10^{-07}$)	$1.1x10^{-06}$ ($5.4x10^{-07}$)
Average CV (SD)			$7.8x10^{-06}$ ($6.6x10^{-06}$)	$1.2x10^{-05}$ ($5.9x10^{-06}$)	$6.7x10^{-06}$ ($3.9x10^{-06}$)	$1.1x10^{-05}$ ($6.5x10^{-06}$)	$7.4x10^{-06}$ ($3.9x10^{-06}$)	$6.6x10^{-06}$ ($4.6x10^{-06}$)	$1.3x10^{-05}$ ($8.5x10^{-06}$)
% of samples > detection limit			92	88	93	76	94	93	93

Table 4.2: Univariate results of the MANOVA comparing elemental concentrations among locations/times for a) embryos and b) juveniles. In addition, results are provided of a nested MANOVA, assessing c) ontogenetic variability in elemental concentrations between embryos and juveniles, which are nested within locations/times of collection (*). Only elements with significant differences are included.

Effect	df	SS	MS	F	p<	SS	MS	F	p	SS	MS	F	p	SS	MS	F	p
A) Embryo																	
		Mn				Zn				Ba				Ce			
Location/time	2	3.2	1.6	13	0.001	100	50	155	0.001	6.4	3.2	27	0.001	3.2	1.6	16	0.001
Error	197	24	0.1			63	0.3			22	0.1			24	0.1		
B) Juvenile																	
		Zn				Sr											
Location/time	2	0.7	0.3	4.2	0.05	0.04	0.02	4.1	0.05								
Error	36	2.9	0.1			0.18	0.01										
C) Ontogenetic analysis																	
		Mn				Zn				Sn				Ba			
Location/time	2	0.7	0.3	3.2	0.05	9.1	4.5	16	0.001	0.003	0.001	0.01	NS	0.8	0.4	3.4	0.05
Embryo vs. Juvenile*	3	79	26	247	0.001	78	26	92	0.001	75	25	226	0.001	88	29	267	0.001
Error	233	25	0.1			66	0.3			26	0.1			26	0.1		
		Ce				Pb											
Location/time	2	1.7	0.9	6.8	0.01	0.3	0.1	1.2	NS								
Embryo vs. Juvenile*	3	107	36	285	0.001	3.6	1.2	11	0.001								
Error	233	29	0.1			25	0.1										

Figure 4.1: Map of study locations. Turneffe Atoll. Belize and Banco Chinchorro. Mexico and sites of embryo and juvenile collections.

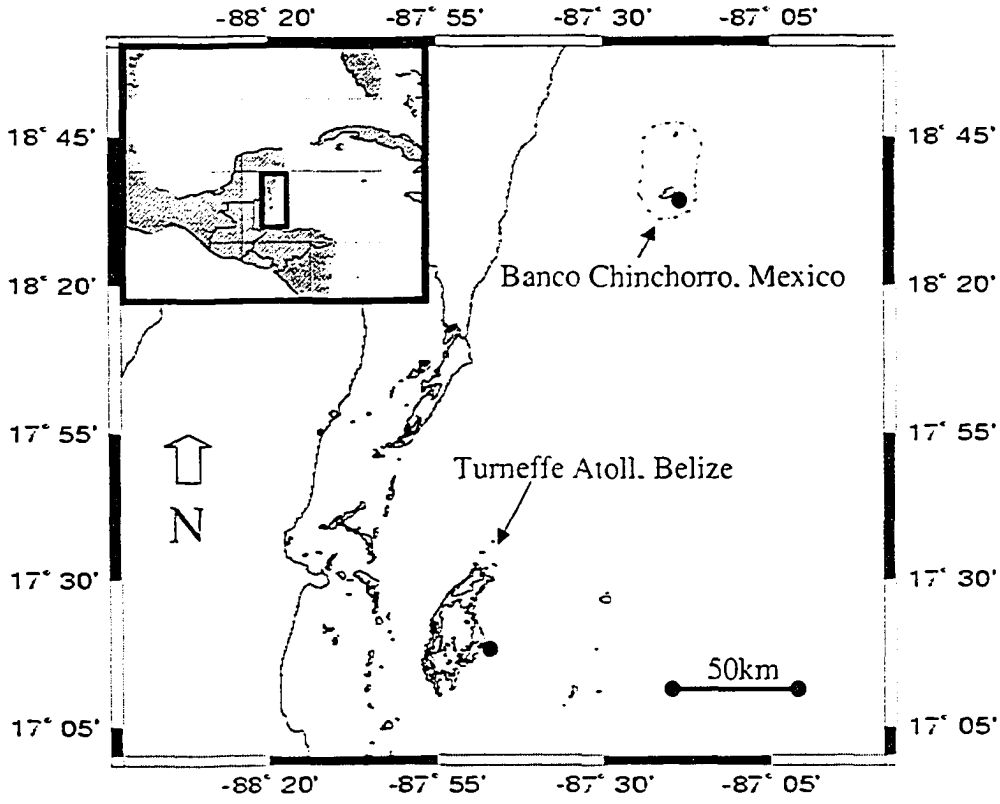
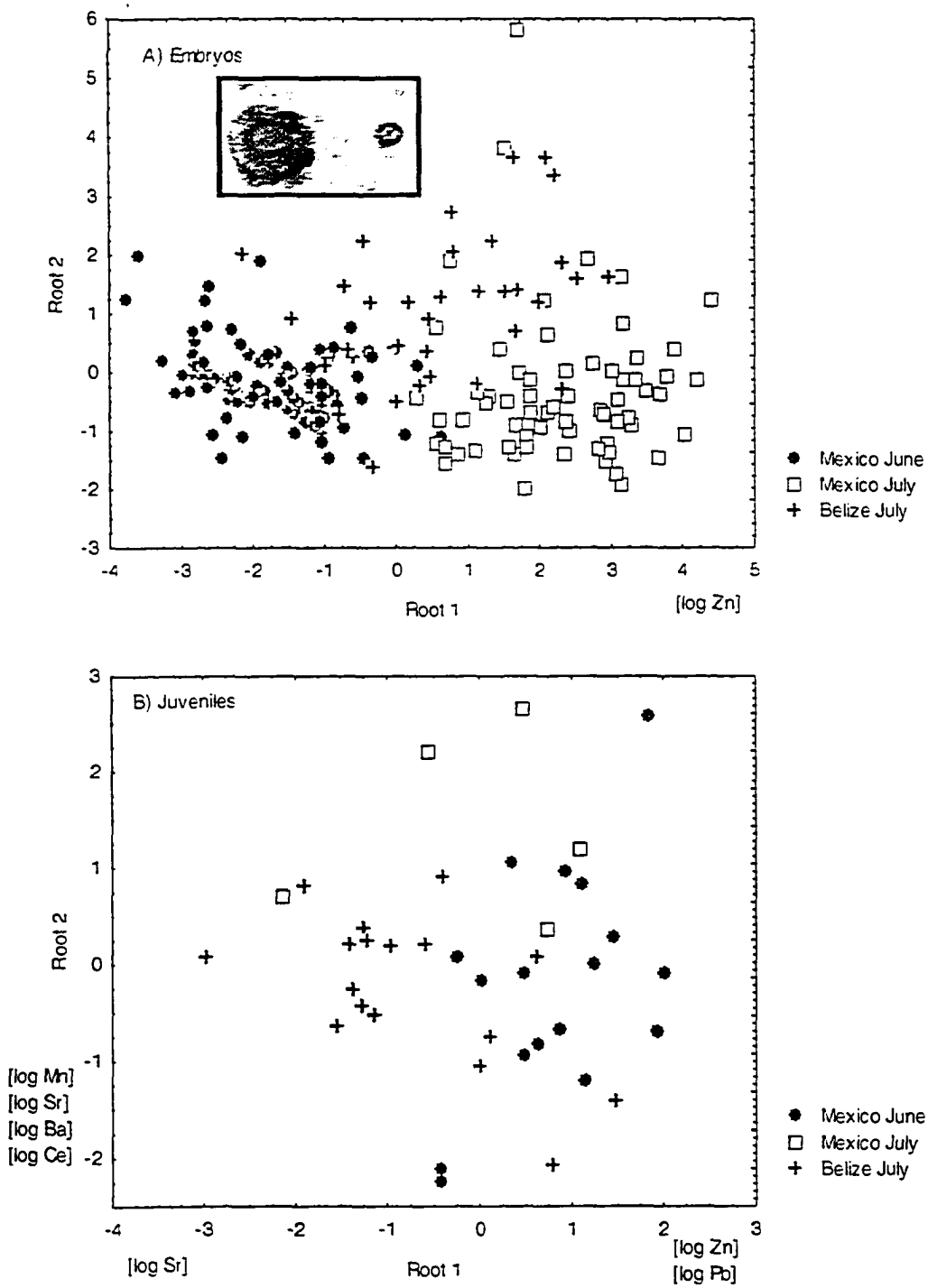


Figure 4.2: Plots of embryo (a) and juvenile (b) fish from their sampling locations/times in DFA space using elemental concentrations of their otoliths. Elements are indicated that account for > 10% of the separation. Although root 2 of figure B was non-significant, it was included for illustrative purposes. Inset is an image of the laser (left oval) approaching a ~14 μm diameter embryo otolith (right circle).



Section B: Demersal stage connectivity

Chapter 5: Discrimination of *Haemulon flavolineatum* from mangrove and coral reef habitats and an assessment of connectivity.

A modified version of this chapter was published as:

P.M. Chittaro, B.J. Fryer, and P.F. Sale.2004. Discrimination of French grunts (*Haemulon flavolineatum*, Desmarest, 1823) from mangrove and coral reef habitats using otolith microchemistry. Journal of Experimental Marine Biology and Ecology. 308: 169-183.

Abstract

French Grunts (*Haemulon flavolineatum*) were held captive in Great Exuma, Bahamas and at Turneffe Atoll, Belize, to investigate whether a) otolith microchemical differences existed between mangroves and reefs separated at biologically relevant spatial scales (0.25 to 7.1 km); b) patterns in elemental concentrations were consistent across years; and c) the movement of fish from mangroves to reefs could be detected. Significant differences in otolith edge chemistry were detected among fish held in mangrove and coral reef habitats in Bahamas (2001) and Belize (2001 and 2002), such that, on average, 77%, 68%, and 85%, respectively, of the individuals were correctly classified to the site they were held. There was considerable temporal variability in the otolith chemistry of Belize fish from 2001 to those from 2002, since only 42%, on average, of fish from 2002 were correctly classified to their captive sites using chemical information from 2001. Finally, to identify whether fish occupied a mangrove as juveniles, we ablated the juvenile portion of the otolith of fish taken from reefs in Belize, 2002. Results indicated that 36% of 39 individuals had an elemental signature more representative of one of the mangrove sites (out of three possible sites), therefore suggesting that mangroves contribute to reef fish populations.

Introduction

Mangroves are one of the most productive environments in the world (Thollot 1992; Beck et al. 2001), but like so many habitats, they are under continual anthropogenic degradation, which in this case has led to a 50% reduction in their global distribution (Ronnback 1999). Although little direct evidence exists it is generally accepted that mangroves function as nursery habitats for juveniles of many reef fish and invertebrates, which are believed to eventually supplement nearby adult populations (usually associated with coral reefs) (Rooker and Dennis 1991; Gillanders and Kingsford 1996; Nagelkerken 2000a; Beck et al. 2001; Gillanders et al. 2003; Heck et al. 2003). Despite its relevance to fisheries conservation and management throughout tropical regions, a detailed understanding of the role mangrove habitats play in structuring reef populations (i.e., direct evidence of the proportion of reef dwelling organisms that occupied a mangrove habitat during their juvenile period) remains elusive.

Fortunately, various methods exist to quantify the movement of organisms. For instance, techniques have been used that employ artificial tags, such as floy and telemetry tags (Hyland et al. 1984; Buckley et al. 1994; Hines et al. 1995; Koutsikopoulos et al. 1995) or natural tags like stable isotopes and trace elements found in various strucutes, such as otoliths, vertebrae, and scales (e.g., Thresher et al. 1994; Thorrold et al. 2001; Wells et al. 2003; and see review by Campana and Thorrold 2001). For fish, only the natural chemical tag found in otoliths provide a temporal record of the environment an organism has occupied (that is metabolically inert as opposed to that of vertebrae and scales; see Campana and Thorrold 2001), while not involving the time or expenses more common to approaches that use artificial tags.

Various studies have used otolith microchemistry to address topics related to fish movements, such as migratory patterns of temperate and tropical marine and freshwater fish (Milton et al. 1997; Thorrold et al. 1997; Dove and Kingsford 1998; Gillanders and Kingsford 2000; Kafemann et al. 2000; Kingsford and Gillanders 2000; Milton et al. 2000; Volk et al. 2000; Secor et al. 2001; Rooker et al. 2003) and patterns of larval dispersal (Jones et al. 1999; Dibacco and Levin 2000). Of these studies the majority have been conducted at spatial scales of 10s of km or greater, and in coastal environments rich in non-carbonate sediments (see Thorrold et al. 1998; Milton et al. 2000). However, a few studies have reported chemical differences in the otoliths of fish collected from sites separated by smaller distances. For instance, differences in otolith microchemistry were detected among collections separated by 0.05 to 6 km for *Parma microlepis* (White-ear Scalyfin) and *Pelates sexlineatus* (Six-lined Trumpeter) (Dove and Kingsford 1998; Kingsford and Gillanders 2000 and Gillanders and Kingsford 2000, respectively). Yamashita et al. (2000), used variability in otolith microchemistry among reefs and estuaries (separated by less than 10 km) to elucidate nursery related habitat use in *Platichthys bicoloratus* (Stone Flounder). They found that 65 and 32% of adults (i.e., 13 and 7 individuals out of 20 and 22, respectively) collected from inshore reefs in 1994 and 1995, respectively, originated from a nearby estuary, and thus suggested that these habitats were important in maintaining nearby populations even though the contribution varied temporally. Also working at the same spatial scale, Gillanders and Kingsford (1996) used otolith microchemistry differences to determine that *Achoerodus viridis* (Blue Grouper) recruited to both nursery (i.e., estuarine seagrass, 41% of individuals) and non-nursery habitat (i.e., rocky reef, 59% of individuals).

If we are interested in using otolith microchemistry to determine whether juvenile reef fish occupy mangroves, it is necessary to first assess whether detectable trace elemental differences exist at biologically relevant spatial scales (i.e., scales at which mangroves and reefs are separated, yet still permit connectivity; meters to kilometers). However, because fish are not fixed in space, their natural movements will likely result in their residence in different areas and/or habitats (e.g., sand, seagrass, mudflat, algal flats etc.) that likley differ in environmental chemistry. Consequently, because of the use of multiple habitats by the same fish over potentially short time scales, our ability to identify elemental differences among habitats may be reduced. Therefore, in order to determine whether spatial variability in otolith microchemistry exists at biologically relevant spatial scales and ensure no confounding effect related to movement among habitats, fish would need to be restricted to a particular habitat (e.g., coral reef or mangrove).

Using *Haemulon flavolineatum* (French grunt; Class Actinopterygii, Order Perciformes, Family Haemulidae), a common Caribbean reef fish known to occupy mangroves (as juveniles) and coral reefs (as juveniles and adults) and suspected to move between them (Nagelkerken 2000), we confined their movements to specific habitats in order to address a series of objectives. Specifically, we were interested in determining whether a) otolith microchemical differences of enclosed *H. flavolineatum* exist between mangroves and reefs separated at a biologically relevant spatial scales (0.25 to 7.1 km) in oceanic locations (Turneffe Atoll, Belize and Lee Stocking Island, Bahamas) remote from most land-derived sediments; b) patterns in elemental concentrations are consistent across years; and c) it is possible to identify if a given fish occupied a mangrove as a juvenile. We anticipate that the findings of this study will provide new insight into the

use of otolith microchemistry for assessing the role mangrove habitats play in structuring populations of reef fish.

Materials and methods

Fieldwork was conducted at Lee Stocking Island, Great Exuma, Bahamas, in May 2001 and Calabash Caye, Turneffe Atoll, Belize, in August 2001 and 2002. Both locations were relatively isolated from coastal or on-shore influences such as pollution and terrigenous sediments. Three sites were established in each of Bahamas and Belize, such that the former had two reef and one mangrove site and the latter had one reef and two mangrove sites (each site encompassed a total area of approximately 200 m^2) (Figure 5.1). Issues pertaining to fish collection and captivity limited the number of sites used in this experiment.

Mangrove sites were 0.75-1.5 m in depth, while reef sites varied between 2 m (Bahamas reef B and Belize reef) and 7m (Bahamas reef A). Bahamas reef A was near a channel opening directly to deep ocean water while reef B and mangrove sites were located on the Exuma bank approximately 6-7 km (linear distance) away from deep water, and separated from each other by a little over 1 km. The reef site in Belize was in the back-reef, but within 0.5 km of deep water. Mangrove A in Belize was situated approximately 0.27 km inshore from the reef site in a mangrove forest that faced the back-reef, while mangrove B was 0.76 km away from the reef site and somewhat isolated within a mangrove channel.

At all sites 15-20 juveniles and sub-adults (7.0-14 cm standard length) of *H. flavolineatum* were collected (using 5 m by 2 m monofilament barrier net, 1 cm stretched mesh) and distributed among 3-4 replicate 1 m^3 enclosures that were completely

submerged on site and separated by a minimum of 5 m. Collecting fish from mangroves in Belize was logistically difficult, and as a result fish were removed from the reef site and placed within enclosures at all three sites. Fish were held within enclosures for 12-14 days during which time they were fed equivalent portions of *Selar crumenophthalamus* (Bigeye shad) daily. Captivity ensured that microchemistry at the otolith edge reflected that of the site where fish were held. Therefore, any discrimination of fish based on their otolith microchemistry would be attributed to site differences in chemistry and not confounded by fish movement to other areas. Although Belize fish in mangrove and reef enclosures were originally collected from the same reef this did not compromise the experiment since we were only interested in the microchemistry at the otolith edge, which corresponded to the two weeks in enclosures. Immediately following the captivity period, standard length and weight were measured for each fish and sagittae were removed and stored dry in individual vials.

Sagittae were embedded in epoxy resin (Gougeon®) and sectioned in a transverse plane using a low speed diamond saw (Buehler®), to a width of 300 μm. Sections were cleaned in an ultrapure hydrogen peroxide bath for 1.5 minutes, rinsed for 2 minutes in milli-Q water, and dried in a laminar flow HEPA filtered fume hood. After cleaning, otoliths were mounted on a microscope slide and stored vertically until microchemical analysis.

Otoliths were chemically analyzed at the Great Lakes Institute of Environmental Research (GLIER), University of Windsor, using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). For fish collected from Bahamas, the ICP-MS used was a high sensitivity Thermo Elemental PQ3(S). This was upgraded to a Thermo

Elemental X7 ICP-MS for analysis of fish collected from Belize. These instruments have similar sensitivity (400 M cps/ppm for mid mass analytes in solution mode) but the X7 has much lower backgrounds (0.5 cps at high mass. vs. 20 cps for the PQ3), resulting in greatly reduced detection limits. Samples analyzed with the PQ3 were not reanalyzed using the X7 ICP-MS since comparisons among locations were not planned. Both ICP-MSs were operated at low resolution with argon used as the carrier gas from the laser sampling cell. The laser sampling system is a purpose-built system (Fryer et al. 1995) based on a non-homogenized, high power, frequency quadrupled (266nm) Nd:YAG (neodymium-doped yttrium aluminum garnet) laser. The laser beam is focused onto the sample using an Olympus® BX-51 petrographic microscope and an Optics For Research® 266 nm 10 X objective lens. We used a 2 mm pinhole beam constrictor to increase the spatial resolution of the laser sampling. The sampling system is more fully described in Crowe et al. (2003).

Portions of interest (i.e. two week period of captivity) on each otolith were targeted using an automated microscope stage. To increase sensitivity, a contour was ablated along the edge of the otolith that was approximately 60-90 μm in length. Data acquisition lasted 180 seconds with 60 seconds of background counts at the start of each ablation. Trace element doped glass standards (National Institute of Standards and Technology 610, a homogenous glass that is spiked with a range of elements of known concentrations) were analyzed at the beginning and end of each sample set to correct for instrument drift. Calcium was used as an internal standard to compensate for signal variation caused by differences in mass of ablated material.

Because of difficulty in identifying otolith daily bands (since all fish were between 1 and 1.6 years of age, and therefore daily rings were compressed) we estimated otolith growth for the two-week captivity period by using daily increment (daily increment formation validated by Brothers and McFarland 1981) widths of 0.7 μm for *H. flavolineatum* juveniles (of approximately 3.5 cm standard length) determined by Brothers and McFarland (1981). We assumed daily increment growth would not significantly decrease at ages reflected in our collections and therefore 12-14 days of captivity corresponded to approximately 8.4-9.8 μm of otolith edge growth. Although the laser was focused to provide a 8-10 μm sampling site it was still possible that ablated material from outside the captivity period would be included. However, the otolith chemistry from Bahamas enclosed fish was assumed to still be an accurate reflection of the environment since fish were collected and held in the same habitat. On the other hand, in Belize, since fish were collected from the reef site and distributed among all sites, any ablated material from outside of the captivity period would bias towards a more "reef" signature. This would therefore minimize microchemical differences among sites, resulting in a conservative estimate of microchemical variability.

For the Belize fish collected in 2002, we microchemically sampled the juvenile portion of each otolith because this could be related to 2001 data. The juvenile portion ablated was approximately 35-60 μm following the settlement mark (i.e. 13-22 days after settlement according to daily increment growth estimates of 2.7 μm by Brothers and McFarland 1981). This portion of the otolith was confirmed by growth curves by Billings and Munro (1974) to represent deposition in 2001.

In total, 23 elements were analyzed by the ICP-MS and chemical concentrations (parts per million) were calculated using Lamtrace software (van Achterbergh et al. 2001). Elements that met the following two criteria were included in statistical analyses: 1) concentrations of NIST samples were determined with a satisfactory precision (coefficient of variation less than 10%); and 2) concentrations in otoliths were three times the detection limit for more than 50% of otoliths analyzed. Three data sets were used: one for each of Bahamas 2001, Belize 2001 and Belize 2002. Each data set was composed of a unique suite of elements defined using the above criteria. All data were $\log_{10}$ transformed to improve normality for the multivariate analyses described below.

One factor analysis of covariance (ANCOVA) followed by Bonferroni post hoc tests were used to determine significant differences in trace element composition of fish taken from mangroves and reefs in Belize (2001 and 2002) and Bahamas (2001). Since there has been some indication that trace element incorporation is related to growth, size, and age of the fish (Begg et al. 1998), the interpretation of spatial variation may be confounded if fish are of different ages and or sizes. Therefore, to reduce any influence of fish size, fish standard length was used as a covariate.

Direct discriminant function analysis (DFA), which maximizes the among-group variation relative to the within group variation (McCune and Grace 2002), was used to determine if the site in which a fish was held could be accurately separated from other sites (only three groups to assign fish) based on the elemental concentrations detected at the edge of the otolith. A separate DFA was performed for each of Bahamas 2001, Belize 2001 and Belize 2002 data. Since the same experiment was conducted in Belize in both 2001 and 2002, we questioned whether the discrimination of fish from sites in 2001

could accurately classify those from 2002, and thus give an indication of temporal variability in otolith microchemistry. To do this, we used classification functions produced from the 2001 DFA as the training dataset to determine the site to which fish from 2002 were classified (using elements that are common to both 2001 and 2002). Finally, to assess the relative contribution of mangrove fish to the reef population, we used functions produced from the 2001 DFA (training dataset) to classify the otolith chemistry from the juvenile portion of fish collected from reefs in 2002 (test dataset).

Elements important in each DFA are indicated by factor correlations greater than |0.33|, which represents approximately 10% of the variance (Tabachnick and Fidell 1996). For each DFA, classification functions were produced that are used to indicate the percentage of individuals from each site that are correctly classified.

Results

Based on the two criteria (coefficient of variation less than 10% for NIST samples and concentrations in otoliths three times greater than detection limit for more than 50% of otoliths analyzed), various elements were retained for statistical analysis. Specifically, Sr and Ba were examined for the Bahamas data set, while Sr, Ba, Pb, and Sn were used for the 2001 Belize dataset, and Sr, Ba, Pb, Sn, Li, Mg, Cu and Rb were included for the 2002 Belize data set (Table 5.1).

ANCOVAs comparing elemental composition of fish from mangrove and reef sites indicated significant differences for certain elements. Specifically, in Bahamas, otolith Sr concentrations were significantly greater for fish from reef than mangrove sites, with the more distant reef A being significantly greater than reef B, while in Belize, 2002, Cu and Sn concentrations were significantly greater in mangroves than reefs (Figure 5.2).

Patterns in otolith concentrations among habitats were not consistent between regions or years (i.e., no overall "mangrove" or "reef" signatures were evident). For example, in Belize, 2001 fish from the reef site had significantly lower Sr concentrations then either mangrove A or B, while in 2002, Sr concentrations differed only among mangrove A and B.

DFA of fish among the three Bahamas sites (2001) indicated highly significant discrimination (Wilks' Lambda = 0.29; $F(4, 82) = 17.07$; $p < 0.001$) that produced one significant function (chi-squared statistic = 50.26, df = 4, $p < 0.001$) explaining 40% of the variability. Factor correlations were -0.89 and 0.34 for Sr and Ba, respectively. The DFA indicated a unique otolith microchemical signature for each site that permitted the correct classification of 73-80% of the individuals (Table 5.2). A level of spatial correlation in otolith microchemistry was apparent since incorrectly classified individuals that were collected from reef B or the mangrove site were most often classified to the adjacent site as opposed to the more distant reef A. However, of the four misclassified individuals collected from reef A, three were grouped with reef B suggesting a habitat correlation in otolith microchemistry. Reef A was discriminated from both reef B and the mangrove site along function 1, with reef A showing higher concentrations of Sr but lower concentrations of Ba relative to the other sites (Figure 5.3a).

DFA of fish among the three Belize sites of 2001 indicated highly significant discrimination (Wilks' Lambda = 0.45; $F(8, 76) = 4.69$; $p < 0.01$) that produced two significant functions (chi-squared statistic = 31.69, df = 8, $p < 0.001$) exaplaing 33% of the variability. Factor correlations for Sr (function 1) and Pb (function 2) were 0.88 and 0.56, respectively. The DFA indicated that there was a unique otolith microchemical

signature for each site that permitted the correct classification of 64-73% of the individuals (Table 5.2). According to the misclassification of individuals from mangrove A and B, there was little influence of spatial or habitat correlation. Along function 1, individuals from mangrove A and B had slightly higher concentrations of Sr relative to the reef, while along function 2, mangrove B tended to have slightly higher concentrations of Pb than mangrove A (Figure 5.3b).

DFA of fish among the three Belize sites of 2002 indicated highly significant discrimination (Wilks' Lambda = 0.26; $F(16, 58) = 3.47$; $p < 0.001$) and produced two significant functions (chi-squared statistic = 43.63, df = 16, $p < 0.001$) explaining 56% of the variability. Factor correlations for Cu were 0.47 and –0.59 (function 1 and function 2, respectively); for Rb, 0.46 (function 2); for Sr, 0.33 and –0.44 (function 1 and 2, respectively); for Sn, 0.48 and –0.33 (function 1 and 2, respectively); and for Pb, 0.34 (function 1). The DFA indicated that there was a unique otolith microchemical signature for each site that permitted the correct classification of 73-93% of the individuals (Table 5.2). Incorrectly classified individuals from the reef and mangrove B were classified to the nearest site, mangrove A, again suggesting some degree of spatial correlation in otolith microchemistry. However, incorrectly classified individuals collected from mangrove A, were 75% more likely to be classified to the slightly further mangrove B, suggesting that a mangrove habitat signature may override any similarity due to spatial correlation. Mangrove B was discriminated from mangrove A and the reef site along function 1, with mangrove B showing higher concentrations of Cu, Sn and Pb and lower concentrations of Sr relative to the other sites (Figure 5.3c). Function 2 was useful in

discriminating mangrove A from the reef site, with the former showing higher concentrations of Cu, Sr and Sn and lower concentrations of Rb.

The use of otolith microchemistry determined from fish collected in Belize (2001) to classify fish collected in 2002, and thus assess temporal variability in otolith microchemistry, indicated considerable variaiblity through time. Only 42% of fish from 2001 were correctly classified based on the chemistry from 2001 (Table 5.2). Specifically, 2002 mangrove B had no individuals that were correctly classified using functions produced from 2001, while the reef and mangrove A showed 60 and 73% (6 and 10 individuals), respectively, correct classification of individuals.

The investigation of the relative contribution of mangrove fish to reef populations using 2001 Belize otolith edge chemistry to classify the juvenile portion of otoliths of fish taken from the Belize reef in 2002, revealed that 64% and 36% of the juveniles were assigned to the reef and mangrove A sites, respectively. Although, fish could only be grouped to three possible sites (reef, mangrove A or mangrove B) none of these individuals indicated any similarity to the more sheltered mangrove site (mangrove B).

Discussion

Most otolith microchemical studies have been conducted at relatively large spatial scales, such that collections were separated by at least 10s of kilometers (see Thorrold et al. 1998; Milton et al. 2000; and Yamashita et al. 2000, but see Dove and Kingsford 1998; Gillanders and Kingsford 2000; Kingsford and Gillanders 2000). Yet, in order to directly determine whether otolith microchemical differences exist at spatial scales relevant to the potential movements of fish between mangroves and reefs, it was necessary to work at scales of 100s of meters to kilometers (e.g., Burke (1995), observed

the nocturnal movements of *H. flavolineatum* of approximately 200 m). In this study, we fixed individuals of *H. flavolineatum*, for a short period of time (12-14 days), to mangrove and coral reef sites that were relatively close to each other (0.25-7.1km).

Contrary to observations by Patterson et al. (1999), in which a lack of discrimination of *Epinephelus striatus* (Nassau grouper) was detected among three sites in Exuma Sound, Bahamas, that were separated by approximately 40-100 km; fish from adjacent sites within Bahamas and Belize had unique otolith microchemical signatures that permitted their correct classification (Table 5.2). Our observed differences in elemental concentrations were surprising given the spatial scale of analysis and that both Bahamas and Belize are locations far from sources of non-carbonate sediments. However, the microchemical differences could be attributed to a combination of topography, hydrography, and productivity. For instance, two of the three reef sites were located adjacent to openings to deep water, and thus likely predisposed to upwelling processes that could transport trace element laden water. In contrast, the shallow and relatively stagnant mangrove waters of the other sites are known to be areas of high primary productivity (Beck et al. 2001), which could distort elemental concentrations relative to the other sites. In fact, Cu and Sn showed lower concentrations on the reef in Belize (2002) with greater concentrations observed moving towards more sheltered mangrove B (Figure 5.2). Regardless of the cause of variability in otolith microchemistry, there was little evidence to suggest a habitat-specific chemical signature based on the examined elements. In other words, a 'mangrove signature' or 'reef signature' was not apparent from the relatively small number of sites that were sampled.

Despite the relatively high levels of spatial discrimination, we found only limited temporal consistency in otolith microchemistry between 2001 and 2002 at Belize sites. Others have also noted limitations in the ability to discriminate populations as a result of temporal variation in otolith microchemistry. For instance, Thorrold et al. (1998) reported that the ability to correctly classify *Alosa sapidissima* (American Shad) to three rivers deteriorated 15% (from 95% to 80%) when classification functions were used on fish collected only two months later. Gillanders and Kingsford (2000) noted significant differences among individuals of *Pelates sexlineatus* (Trumpeter) collected within and among estuaries, but since there was a time interaction, they suggested that chemistry would need to be determined each year if it is to be used to determine the estuary fish recruited to. Similarly, Campana et al. (2000) observed only short-term temporal stability (up to 1 year) in the microchemical signature of *Gadus morhua* (Atlantic cod) and therefore suggested that otoliths could serve only as seasonal tags for specific groups. However, temporal persistence was noted between two year-classes of *Thunnus thynnus* (Blue fin tuna) (Rooker et al. 2003) and between *Aldrichetta forsteri* (Yellow-eye mullet) collected at the same sites in 1986 and 1988 (Edmunds et al. 1992).

By comparing the successful spatial discrimination of habitats (determined from the otolith edge chemistry) to the chemistry of the juvenile portion of the otolith (both of which correspond to the same time), we investigated the contribution of fish from mangrove to coral reef habitat. Results indicated that approximately 36% of the individuals collected from the reef site had spent their juvenile life at sites resembling mangrove A. Interestingly, Forrester and Swearer (2002), working along southern California, classified 11 and 8 individuals (i.e., 58% and 42% of 19 sampled individuals,

respectively) of *Paralichthys californicus* (California halibut) as having utilized a bay and open coast nursery, respectively. In addition, Gillanders and Kingsford (1996) woking in southeastern Australia, observed a similar pattern of habitat use, such that 41% of *Achoeroclus viridis* recruited first to estuarine seagrass while 59% recruited directly to rocky reefs. However, as was noted by Gillanders and Kingsford (1996), our study was also greatly constrained by the analysis, since fish could only be grouped to three possible sites. It is likely that a site not represented may better classify individuals, thus altering our estimates of mangrove-reef connectivity.

We have demonstrated that otolith microchemistry of *H. flavolineatum* held experimentally within closely associated mangrove and reef sites was distinct enough to permit their accurate assignment to sites. However, due to the detected temporal variation in otolith microchemistry it appears that it will be necessary to re-calculate site "signatures" using fish known to be resident on each occasion. The findings of this study also highlight that mangroves may, to some degree, contribute fish to reef populations, yet more work is required (i.e., a greater number of sites, habitats and time periods, as well as a study of wild fish) to accurately quantify the movement among habitats and thus begin to truly understand the relative importance of mangroves as nurseries.

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Table 5.1: Isotopes included in the DFA for Bahamas and Belize determined from coefficient of variation (CV) of NIST samples being < 10% and > 50% of otolith samples greater than three times detection limit. Average and standard deviation provided for detection limit by sample ablation and concentration (ppm). Average CV is the mean of CV of 5 (Belize 2002) or 6 (Bahamas 2001 and Belize 2001) different sets of otolith ablations. All values in parts per million.

Location/time	Isotope measured	Average. CV (SD)	% of samples > 3X detection limit	Average detection limit by sample (SD)	Average concentration (SD)
Bahamas 2001					
	Sr (86)	2.62 (2.16)	98	1.02 ± 0.13	3206 (1038)
	Ba (138)	3.07 (2.99)	97	0.44 ± 0.05	3.09 (1.22)
Belize 2001					
	Sr (86)	4.99 (4.14)	100	1.28 ± 0.68	3098 (582)
	Ba (138)	5.84 (2.52)	100	0.55 ± 0.03	3.52 (1.73)
	Sn (120)	5.42 (2.37)	79	0.15 ± 0.07	1.13 (1.12)
	Pb (208)	6.43 (2.43)	78	0.11 ± 0.10	0.39 (0.62)
Belize 2002					
	Sr (86)	3.52 (2.31)	100	0.42 ± 0.19	2730 (319)
	Ba (138)	4.30 (3.29)	85	0.01 ± 0.02	4.46 (2.44)
	Sn (120)	4.71 (3.41)	83	0.10 ± 0.03	1.52 (1.87)
	Pb (208)	7.18 (4.37)	50	0.04 ± 0.05	0.07 (0.07)
	Li (7)	4.16 (2.06)	100	0.01 ± 0.01	0.16 (0.03)
	Mg (25)	5.52 (1.21)	100	0.05 ± 0.11	15.54 (4.96)
	Cu (65)	4.30 (2.21)	88	0.15 ± 0.55	2.03 (4.59)
	Rb (85)	5.27 (2.91)	62	0.12 ± 0.01	0.05 (0.04)

Table 5.2: Classification matrix for the spatial analysis of each of Bahamas 2001, Belize 2001 and Belize 2002, from their respective DFA. Also included are the DFA results of the assessment of temporal variability (i.e., temporal analysis) in otolith microchemistry as well as the estimation of fish movement between mangrove and reef sites (i.e., connectivity analysis). The temporal variability in microchemistry attempted to classify fish collected in 2002 based on the DFA produced from Belize reefs in 2001 data. For the connectivity analysis, the otolith chemistry for the juvenile portion of fish collected from reefs in Belize 2002 were compared to the otolith edge chemistry of fish from the three sites in 2001.

Site individuals collected from	% correct	Site individuals classified to			n
Spatial Analysis					
Bahamas 2001		Reef A	Reef B	Mangrove	
Reef A	73	11	3	1	15
Reef B	80	1	12	2	15
Mangrove	80	0	3	12	15
Average	78				
Belize 2001		Reef	Mangrove A	Mangrove B	
Reef	66	10	3	2	15
Mangrove A	64	2	9	3	14
Mangrove B	73	3	1	11	15
Average	68				
Belize 2002		Reef	Mangrove A	Mangrove B	
Reef	90	9	1	0	10
Mangrove A	73	1	11	3	15
Mangrove B	93	0	1	13	14
Average	85				
Temporal analysis		Reef 2001	Mangrove A 2001	Mangrove B 2001	
Reef 2002	60	6	4	0	10
Mangrove A 2002	73	5	10	0	15
Mangrove B 2002	0	5	9	0	14
Average	42				
Connectivity analysis		Reef 2001	Mangrove A 2001	Mangrove B 2001	
Reef 2002		25	14	0	39

Figure 5.1: Location of enclosure sites at Lee Stocking Island, Great Exuma, Bahamas (2 reef and 1 mangrove) and Calabash Caye, Turneffe Atoll, Belize (1 reef and 2 mangrove).

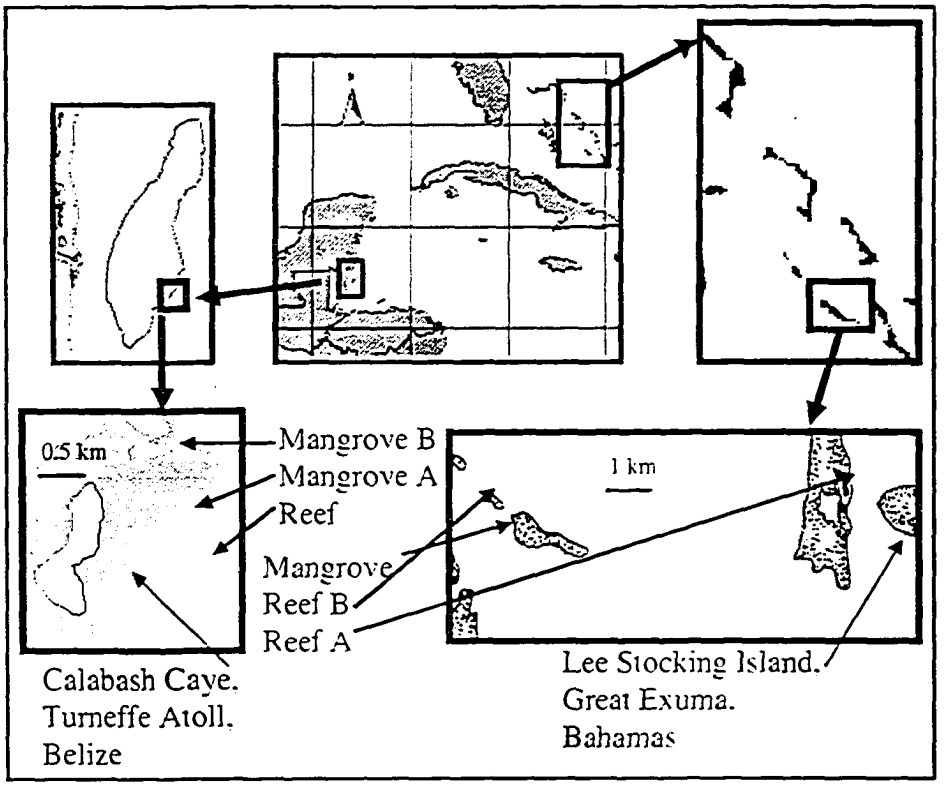


Figure 5.2: Trace element concentrations (ppm) showing differences among sites within a region and year as determined by ANCOVA. Different letters indicate significant differences in pairwise comparisons from Bonferri post hoc test. Analyses were performed using $\log_{10}$ transformed data, but unstandarized data are presented.

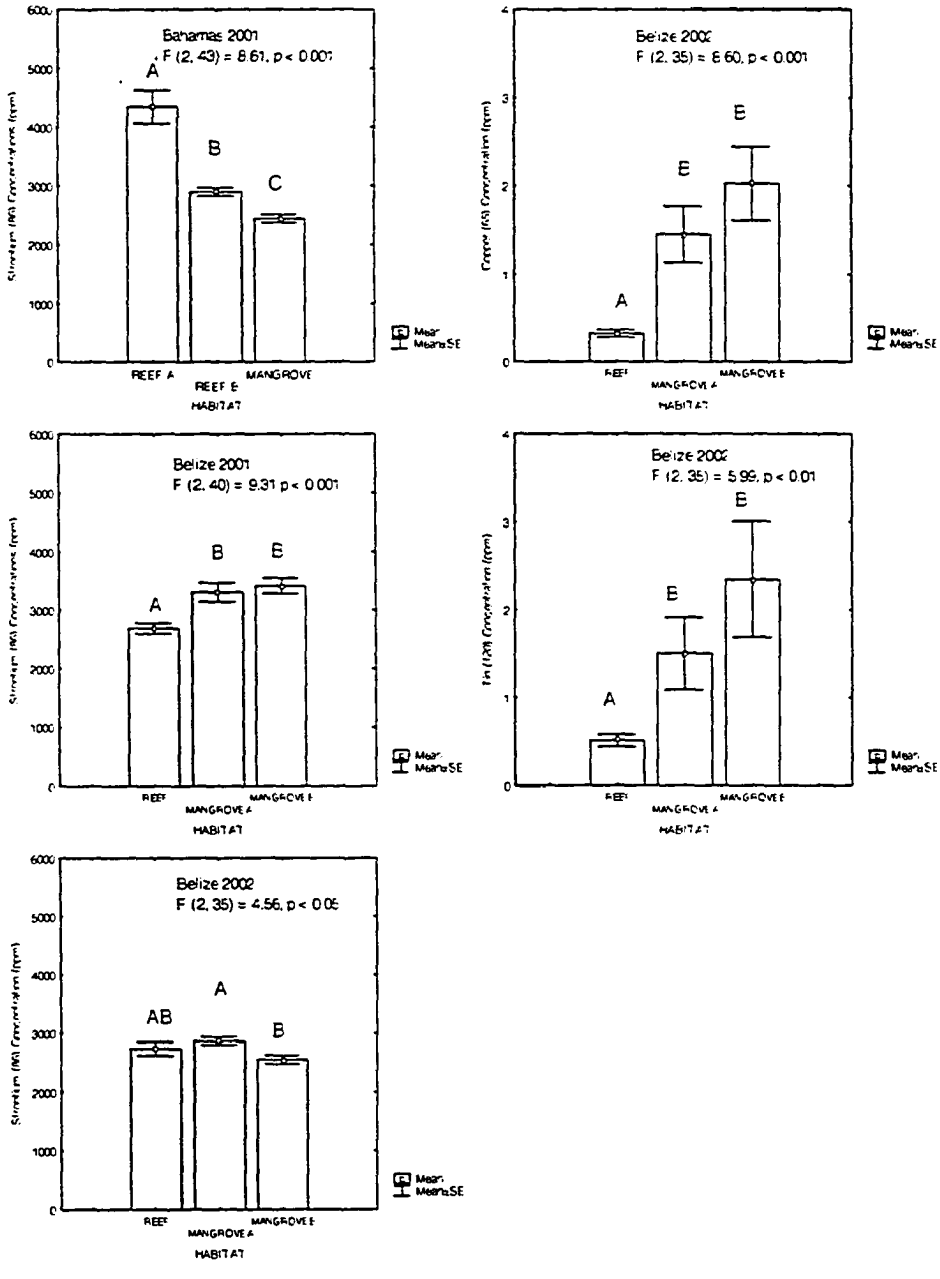
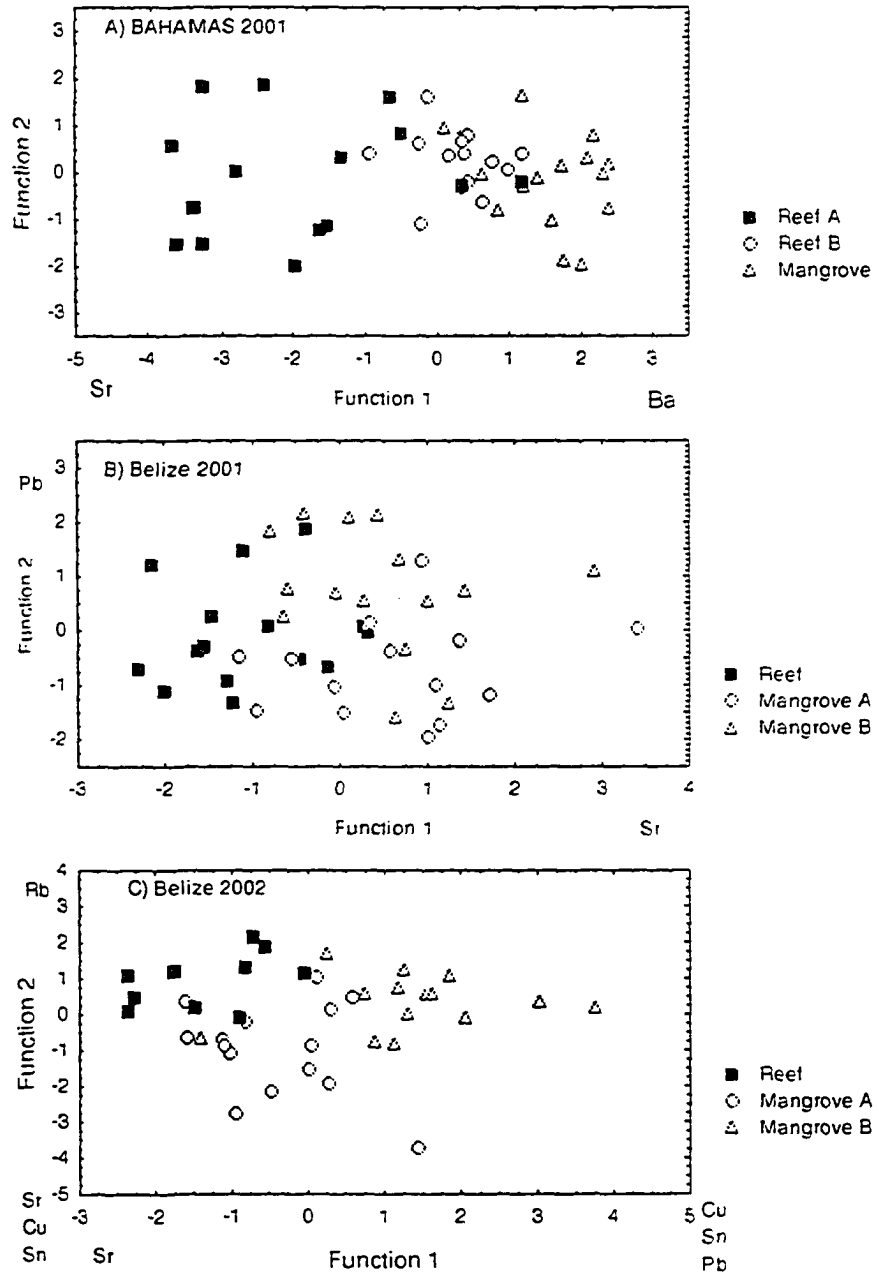


Figure 5.3: Plot of factor scores produced from the DFA of otolith chemistry of fish held at three sites in a) Bahamas 2001, b) Belize 2001, and c) Belize 2002. Elements that account for a substantial amount of variability in the discrimination are indicated. For Bahamas 2001, although function 2 did not significantly improve the discrimination it was included for illustrative purposes.



Chapter 6: Using otolith microchemistry of *Haemulon flavolineatum* (French grunt) to characterize mangroves and coral reefs throughout Turneffe Atoll, Belize: difficulties at small spatial scales.

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Abstract

We investigated whether otolith chemistry of *Haemulon flavolineatum* (French grunt), a nocturnally active fish, could be used as a means to differentiate individuals occupying mangrove and coral reef habitats. In 2003, adults were collected from 9 mangrove (n=65) and 10 coral reef sites (n=97) (sites separated by 0.8-20 km) throughout Turneffe Atoll, Belize. Concentrations of trace elements were measured at the edge of sagittal otoliths by laser ablated inductively coupled plasma mass spectrometry. Results of a two factor nested MANCOVA (sites nested within habitat and covariate of fish size), used to investigate whether significant differences in otolith elemental concentrations existed between habitats (i.e., mangrove versus reef) and among sites, indicated significant differences between habitats, in terms of Li, Mg, Zn and Rb (fish from mangroves had greater concentrations than those from coral reefs), as well as among sites (for several elements). Because elemental variability existed between habitats and among sites, we asked whether this variability was sufficient to differentiate habitats and sites using separate Discriminant Function Analyses (DFA). Results of the DFAs indicated that fish were classified to the habitat (mangrove or reef) from which they were collected with a moderate degree of accuracy (correct classification of 74% and 79% for mangrove and coral reef fish, respectively), but were poorly classified to the site from which they were collected (average correct classification of 46% with a range of 0-89%). Overall, otolith microchemical investigations of *H. flavolineatum* at Turneffe Atoll can be used to identify movement between habitats, yet due to the lack of unique site-specific chemical signatures, likely a result of the nocturnal movement of individuals, it will not be possible to identify specific sites from which reef fish originated.

Introduction

Shallow water habitats such as mangroves have traditionally been regarded as areas that provide food and shelter for developing fish and crustaceans, as well as sources of recruits for nearby coral reefs (see reviews by Beck et al. 2001; Gillanders et al. 2003; Sheridan and Hays, 2003). The extent of connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals; Mora and Sale 2002) between potential nursery (e.g., mangrove) and adult (e.g., coral reef) habitat is relevant to fisheries conservation and management throughout the world. Although numerous ecological processes, such as competition, predation, and responses to abiotic factors, determine the distribution and abundance of reef fish populations, direct quantification of connectivity remains a particularly significant gap in our understanding. Fortunately, methods exist, such as otolith microchemistry, which have been used to successfully quantify movement of fish among populations.

Because the chemical nature of an otolith reflects the environment in which a fish resides (various elements have shown correspondence between the otolith and environmental concentration: e.g., Sr- Farrell and Campana 1996; Gallahar and Kingsford 1996; Bath et al. 2000; Milton and Chenery 2001; Kennedy et al. 2002; Eldson and Gillanders 2003; Ba- Bath et al. 2000; Milton and Chenery 2001; Eldson and Gillanders 2003; Li- Milton and Chenery 2001; Pb- Geffen et al. 1998), otolith microchemistry can provide information on habitat use and thus the nursery potential of mangroves. By using otolith trace element concentrations, several studies have successfully investigated the spatial arrangement of fish (e.g., Edmunds et al. 1989; Dove and Kingsford 1998; Patterson et al. 1999; Kingsford and Gillanders 2000; Rooker et al. 2003) as well as the migration of individuals and resulting connectivity among

populations (e.g., Milton et al. 1997; Swearer et al. 1999; Gillanders and Kingsford 2000; Kafemann et al. 2000; Milton et al. 2000; Thorrold et al. 1998; Volk et al. 2000; Yamashita 2000; Secor et al. 2001; Thorrold et al. 2001; Gillanders 2002; Forrester and Swearer 2002).

Many otolith microchemical studies have observed substantial differences in trace element concentrations among sites separated by more than 10 km (see Thorrold et al. 1998; Milton et al. 2000; and see Table 2 in Gillanders et al. 2001), and a growing number of studies have successfully detected differences in otolith microchemistry at smaller distances. For instance, Dove and Kingsford (1998) and Kingsford and Gillanders (2000), working at sites separated by 0.5 to 3 km, reported microchemical differences in the otoliths of *Parma microlepis* (White-ear scalyfin) in Australia. Yamashita et al. (2000) observed microchemical differences in otoliths of *Platichthys bicoloratus* (Stone flounder) from reef and estuary sites separated by 5 km in Japan, and used this spatial variability to identify individuals that originated from estuarine nursery grounds. Similarly, Chittaro et al. (2004; Chapter 5), working among mangrove and reef sites that were separated by distances of 0.25 to 7 km in Belize and Bahamas, reported differences in the otolith microchemistry of caged *Haemulon flavolineatum* (French grunt). Using this chemical variability among habitats, they determined that 36% of 39 individuals taken from a reef had elemental signatures from juvenile portions of their otoliths that were representative of a nearby mangrove site.

In this study, we expand on previous work by Chittaro et al. (2004), to further examine the utility of using otolith microchemistry of *H. flavolineatum* in population discrimination and connectivity between potential nursery and adult habitats. To assess

whether detectable chemical variability existed between adjacent habitats and thus whether otolith microchemistry is a feasible technique to investigate fish movement between habitats. Chittaro et al. (2004) placed *H. flavolineatum* in cages within mangrove and reef sites in Belize and Bahamas (sites were separated 0.25 to 7.1 km). Since *H. flavolineatum* is nocturnally active, the caging experiment ensured that the microchemistry at the otolith edge reflected the site fish were held and thus prevented any confounding effect that may result from fish movement. Results reported by Chittaro et al. (2004) indicated that otolith microchemical differences were sufficient to identify adjacent sites and thus further investigations were encouraged. In this study, we assess the ability to differentiate individuals of *H. flavolineatum*, in the absence of captivity, collected from sites throughout Turneffe Atoll, Belize: 9 mangrove sites and 10 coral reef sites (adjacent sites were separated 0.8 to 20 km). Specifically, we investigate whether it is possible to identify the habitat (i.e., mangrove or coral reef), and at a finer resolution, the sites from which individuals were collected.

Materials and methods

Our sampling location, Turneffe Atoll, Belize, is a large (50 km long and 16 km wide) complex of cayes that are isolated from the mainland (51 km) and the Belize barrier reef (14 km) by a 275-300 m deep channel. Turneffe Atoll is an ideal location for investigations of nursery habitats and movements of fish, since the numerous cayes that form the atoll are covered with an extensive mangrove forest (covering 74.2 km^2), while the perimeter of Turneffe Atoll is made of a barrier reef.

Haemulon flavolineatum is an abundant Caribbean fish (Class Actinopterygii, Order Perciformes, Family Haemulidae) of moderate commercial value, which is known

to occupy mangrove and reef habitat (Billings and Munro 1974: Mumby et al. 2004) and suspected to move between them (Brothers and McFarland 1981: Nagelkerken 2000a and b). Individuals of *H. flavolineatum* (4 to 13 per site) were collected at 19 sites (9 mangrove and 10 reef sites) at Turneffe Atoll, over 20 days (July 21 - August 9, 2003) (Figure 6.1) using both hand spear and gill net (5 m by 2 m monofilament barrier net, 1cm stretched mesh). Coral reef sites were located on back reef sections of the large continuous reef surrounding Turneffe Atoll and were at a depth of approximately 0.75 to 1.5 m. Mangrove sites were chosen based on their accessibility as well as their proximity to coral reef sites (although desired, paired mangrove and coral reef sites were not always possible: e.g., reef site (RF) 1, 7, 8, and 10 did not have a corresponding mangrove site) and were approximately 1 to 3m deep (each site encompassed a total area of approximately 200 m^2). Adjacent sites were separated by 0.8 to 20km's, with most sites separated by >4km (55km separated the most distant sites). Immediately after collection, we measured standard length and removed sagittae, which were stored dry in individual vials.

At the University of Windsor, we embedded sagittae in epoxy resin (Gougeon®) and sectioned them in a transverse plane, using a low speed diamond saw (Buehler®), to a width of 350 μm. In a class 100 clean room we mounted multiple otolith sections (up to 30) to a microscope slide, sonicated in a milli-Q water bath for 2.5 minutes, triple rinsed in 95% ethanol, triple rinsed in milli-Q water, and dried in a laminar flow HEPA filtered fume hood. Otoliths were chemically analyzed at the Great Lakes Institute of Environmental Research, University of Windsor, using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). A Thermo Elemental X7 ICP-MS

was operated at low resolution using argon as the carrier gas. The laser sampling system is a purpose-built system (Fryer et al. 1995) based on a non-homogenized, high power, frequency quadrupled (266nm) Nd:YAG (neodymium-doped yttrium aluminum garnet) laser. The laser beam is focused onto the sample using an Olympus® BX-51 petrographic microscope and an Optics For Research® 266 nm 10 X objective lens. A 1.5 mm pinhole beam constrictor was used to increase the spatial resolution of the laser sampling (beam diameter was approximately 15-20 μm). The sampling system is more fully described in Crowe et al. (2003).

The otolith edge (which corresponds to the site from which fish were collected, but see discussion) was targeted using an automated microscope stage resulting in a contour of approximately 80 to 120 μm in length (speed of the stage varied between 3-5 μm/sec). Data acquisition lasted 100 seconds with 60 seconds of background acquisition at the start of each ablation. Trace element doped glass standards (National Institute of Standards and Technology, NIST, 610, a homogenous glass that is spiked with a range of elements of known concentrations) were analyzed at the beginning and end of each sample set to correct for instrument drift. Calcium was used as an internal standard to compensate for signal variation caused by differences in the amount of ablated material.

In total, 20 isotopes were analyzed by the ICP-MS and chemical concentrations and detection limits (parts per million) were calculated using Lamtrace software (van Achterbergh et al. 2001). Elements that met the following two criteria were included in statistical analyses: 1) concentrations of NIST samples were determined with a satisfactory precision (coefficient of variation less than 10%); and 2) concentrations in otoliths were greater than the detection limit for more than 50% of otoliths analyzed.

Prior to any analysis, we removed outliers for each site if their value was greater than three times the interquartile distance (see Fowler et al.1995; StatSoft 2001). The remaining data were $\log_{10}$ transformed to improve normality for multivariate analyses (see below).

To investigate patterns of elemental concentrations between mangrove and reef habitats we used a multivariate approach and therefore tests of homogeneity of slopes, homogeneity of variance and normality were required. If assumptions were met, then a nested MANCOVA was used with sites (9 mangrove sites and 10 reef sites) nested within habitats. Since trace element incorporation may be related to growth and thus the size and age of fish (Begg et al. 1998), the interpretation of habitat and/or spatial variation may be confounded if fish are of different ages and/or sizes. To reduce any influence of fish size, fish standard length was used as a covariate. Dependent variables were the elemental concentrations and independent variables were habitat (mangrove or reef) and sites. If significant differences among sites within habitats were detected, a Tukeys HSD post hoc test for unequal sample sizes would be used to determine which sites were significantly different from each other.

We performed two discriminant function analyses (DFA): one at the level of habitats and the other at the level of sites. Specifically, one DFA tested whether otolith microchemical differences were substantial enough to differentiate mangrove and coral reef habitat, while the other determined if there was sufficient variability in elemental concentrations to identify the sites (within mangrove and coral reef habitats) from which fish were collected. A classification matrix and partial Wilks' Lambda statistic were determined for both DFAs; the former indicates the percent of fish that were correctly

identified to the habitat/site they were collected from, while the latter indicates the element(s) that explained the greatest degree of separation between/among habitat/sites (StatSoft, Inc. 2001).

Results

Based on the two criteria (concentrations of NIST samples had coefficient of variation less than 10%, and concentrations in otoliths were greater than the detection limit for more than 50% of otoliths analyzed), several elements were retained for statistical analysis. Specifically, Li, Mg, Cu, Zn, Rb, Sr, Sn, Ba, and Pb were at concentrations sufficiently above detection limit to permit meaningful interpretations (Table 6.1). Outlier analysis removed 11 fish from the 173 that were collected, resulting in 65 fish analyzed for mangrove sites and 97 analyzed for coral reef sites.

Since assumptions of homogeneity of slopes, homogeneity of variance, and normality were met, a traditional nested MANCOVA was performed (Sn and Sr showed a lack of normality even after transformation, and therefore were excluded from this analysis). Overall, there were significant differences in elemental concentrations of fish from mangrove and coral reef habitats (Wilks' Lambda=0.10; df=153, 1090; F=2.34; $p<0.001$). We observed statistical significance of the univariate analyses between habitats in terms of Li, Mg, Zn and Rb, such that concentrations of these elements were greater in mangroves than on reefs (Figure 6.2 and 6.3). In addition, we observed significant variability in the concentrations of most elements (Li, Cu, Rb, Sr, Sn, Ba, and Pb) among certain sites within habitats (Figure 6.2 indicates the univariate results of the site comparison for Li, Mg, Zn and Rb, but only Li and Rb were significant). Tukeys HSD post hoc test, revealed only a small number of pair-wise comparisons were

significant (i.e., for Li, Cu, Rb, Sr, Sn, Ba, and Pb, 10, 2, 9, 23, 6, 10, and 4 pair-wise comparisons, respectively, were significantly different out of 171 possible comparisons), and thus a limited spatial pattern in otolith microchemistry was observed. For instance, the highest Li concentration was observed to be at a central reef site (RF4), while concentrations were at their lowest at the northern and southern extremes (RF1 and 8) (Figure 6.3). Otolith concentrations of Mg were relatively high throughout the central portion of the atoll with a slight decrease in concentrations moving from east to west. Concentrations of Zn and Rb were highest at the south-eastern side of the atoll (MG4, 5 and RF 5, 6).

Although Sr and Sn did not meet assumptions of normality we included them in the DFA because this analysis is relatively robust with respect to skew (McCune and Grace 2002). Significant discrimination (Wilks' Lambda = 0.69; df=9,152; F= 7.39; p< 0.001) was observed for the DFA comparing elemental concentrations of fish from mangrove and coral reef habitats, such that one significant function (chi-squared statistic = 56.44, df=9, p< 0.001) was produced explaining 55% of the variation. The partial Wilks' Lambda was relatively high for all elements (the lowest values, 0.93-0.95, were for Zn, Rb, and Sr), therefore suggesting that all elements contributed relatively weakly to the discrimination of fish between mangrove and reefs sites. Overall, the DFA indicated that otolith microchemistry varied sufficiently between habitats to permit the correct classification of 74% and 79% of the fish to mangrove and coral reef habitat, respectively.

Results of the DFA comparing otolith microchemistry among sites indicated significant discrimination (Wilks' Lambda = 0.06; F (162, 1110) = 2.82; p< 0.001), such

that four significant functions (chi-squared statistic for no roots removed = 410.38, df=162, $p < 0.001$) were produced explaining 76% of the variation (first two functions accounted for 58% of the variation). The greatest discriminatory ability for this model (i.e., partial Wilks' Lambda) resulted from Li, Sr, and Pb (0.63 – 0.70). Overall, the DFA indicated a poor ability to correctly classify fish to the site they were collected (average correct classification of 46%; and a range of 0-89%). Misclassified reef fish were most often classified to other reefs sites (60%) than to mangrove sites (40%), while misclassified mangrove fish were most often classified to reef sites (74%) than to other mangrove sites (26%). Although larger site sample sizes are ideal (average number of fish per site was 9.7 and 7.2 for coral reefs and mangroves, respectively), the lowest correct classifications, 0 and 22%, were from sites with low (MG9; 5 fish) to moderate (RF5; 9 fish) sample sizes. No spatial correlation in otolith chemistry was observed since only 8% of misclassified fish (7 of 84 individuals) were classified to adjacent sites (defined here as < 5km).

Discussion

The elemental composition of otoliths of *H. flavolineatum* (French grunt) showed significant variation between mangrove and coral reef habitats. Although it was not possible to classify a quarter of all fish to the habitat from which they were collected, there was sufficient chemical variability to reliably separate habitats and thus provide a generalization of the chemical nature of mangrove and coral reef fish. Specifically, the chemical identification of fish from mangrove and reef habitats was facilitated by Li, Mg, Zn, and Rb, which showed significantly greater concentrations in the former habitat (Fig. 2 and 3) (Cu, Ba, and Pb showed a similar, but non-significant pattern).

Our analysis of otolith microchemistry among sites revealed that it was not possible to identify the site from which individuals were collected. The difficulty in assigning fish to specific sites is appreciated when the otolith concentrations of Li, Mg, Zn, and Rb were plotted spatially and the lack of strong elemental differences observed (see Fig. 3). However, the relatively unique elemental concentrations at four reef (RF2, 5, 7, and 9) and two mangrove (MG2 and 6) sites facilitated the correct classification of more than 62% of their individuals (i.e., 5-6 individuals out of a sample of 9-10) to the site from which they were collected. These unique concentrations may result from the local hydrology. Specifically, three of these reef sites and one mangrove site (RF2, 5, and 9 and MG6) were adjacent to boat channels (i.e., channels wide and deep enough to easily permit the passage of recreational boats), which may have acted as funnels through which larger volumes of trace element laden water would pass and consequently supply the otoliths of nearby fish.

Apart from the differences in otolith elemental concentrations at a few sites (see above), we observed substantial overlap elsewhere, which prevented the separation of collected fish. This limited discrimination was not surprising since the 20 sites were relatively close together (adjacent sites separated by 0.8 to 20 km) and were confined to an area (Tumeffe Atoll) that lacks substantial inputs of terrigenous sediments more common in coastal locations. A similar lack of discriminatory ability was reported by Patterson et al. (1999) for collections of *Epinephelus striatus* from three sites in Exuma Sound, Bahamas (sites were separated 50 to 150 km), as well as Gillanders et al. (2001) for populations of *Diplodus vulgaris* (Two-branded bream) (separated by 100s of m to 10s of km) in the Mediterranean. Both of these studies suggested that the difficulties

discriminating populations were due to the lack of trace elemental sources, such as freshwater inputs and upwelling.

Throughout otolith microchemistry literature, there are many examples of successful discrimination of populations separated by relatively small distances (e.g., < 10 km). However, the majority of these studies have been conducted in coastal systems where sources of trace elements are likely to be more numerous, thus resulting in greater spatial variability in otolith microchemistry. For example, along the coastline of Australia, significant otolith microchemical differences for *Pelates sexlineatus* (Six-lined trumpeter) (Gillanders and Kingsford 2000) and *Pagrus auratus* (Sparidae) (Gillanders 2002 and Hamer et al. 2003) were observed among sites (separated less than 6 km) within estuaries, whereby the elevated concentrations of certain elements were linked to the local geology and pollution (see Dove and Kingsford 1998). Specifically, elevated concentrations of Ba in otoliths of *Parma microlepis* (White-ear scalyfin) and *Pagrus auratus* (Sparidae) were detected in Jervis Bay, within which existed a petroleum storage facility (Dove and Kingsford 1998 and Gillanders 2002, respectively), while the increased levels of Hg at another location (Malabar) was correlated with a nearby sewage treatment plant (Dove and Kingsford 1998). In addition, greater concentrations of Mn at three locations (Terrigal, South Head, and Bundeena) were likely related to the proximity to freshwater input containing agricultural waste such as Mn-laden fertilizers (Dove and Kingsford 1998). Consequently, the ability to discriminate fish at relatively small spatial scales is likely improved when sampling locations proximal to human development and/or areas of specific geology, both of which can influence concentrations of trace elements in the water and in turn within the otolith (de Pontual and Geffen 2002). Yet

without likely sources of pollution nearby. Chittaro et al. (2004), working at very small spatial scales (as small as 0.25 km) at Turneffe Atoll, Belize, and Lee Stocking Island, Bahamas, observed significant differences in experimentally held fish among mangrove and reef sites.

Why was it possible for Chittaro et al. (2004) to discriminate *H. flavolineatum* from specific mangrove and coral reef sites at Turneffe Atoll (68-85% correct classification based on 3 sites), yet not for this study (46% correct classification based on 20 sites), the latter of which encompassed a much larger spatial scale (the majority of sites were separated by >4 km) with an expected greater variability in elemental concentrations? Apart from the lack of terrigenous inputs at Turneffe Atoll relative to that of more coastal locations (as discussed above), we suspect that an important factor influencing the lack of discriminatory power among sites for this study (relative to that of Chittaro et al. 2004) resulted from fish movements that were unavoidably incorporated into the portion of the otolith targeted for LA-ICP-MS analysis. Specifically, *H. flavolineatum* is a nocturnal predator that migrates into surrounding habitats (e.g., sandy areas) to forage on benthic invertebrates (Burke 1995), and therefore any distinct elemental signature from their diurnal habitat and site (for example a specific mangrove or coral reef site) would be supplemented by new otolith growth that incorporated the chemistry resulting from their nocturnal movement. Because of this movement and the incorporation of elemental signatures from other areas, the variability in otolith elemental concentrations for fish from a given site would increase resulting in difficulties discriminating fish collected from different sites. Further complicating matters, is that the distance traveled during nocturnal foraging may vary among individuals (*H.*

flavolineatum have been observed to remain relatively stationary or move up to 199 metres away; see Burke 1995), which would again increase the variability in otolith elemental concentrations of fish collected from the same site, and thus make it more difficult to differentiate fish collected from multiple sites. Therefore, to avoid this confounding influence of nocturnal movement, Chittaro et al. (2004) held *H. flavolineatum* in enclosures to ensure the chemical signature was from a known site. Consequently, the discrimination that they reported was a result of the experimental design (i.e., a limitation to the movement of *H. flavolineatum*) and not necessarily, what would have been observed if *H. flavolineatum* were allowed to conduct their natural nocturnal migrations.

Overall, this study determined that Li, Mg, Zn, and Rb characterized *H. flavolineatum* collected from mangrove and coral reefs sites, and therefore suggests that these elements would be useful in examining movements between habitats throughout Turneffe Atoll, Belize. However, because of the confounding effects of nocturnal fish movements, combined with the limited inputs of terrigenous sediments at Turneffe Atoll, site-specific variability in otolith microchemistry was not sufficient to classify fish to their site of collection. Consequently, otolith microchemistry is an effective technique that can be applied to assess movement of individuals between mangrove and coral reef habitats, but not at a finer spatial resolution at this location.

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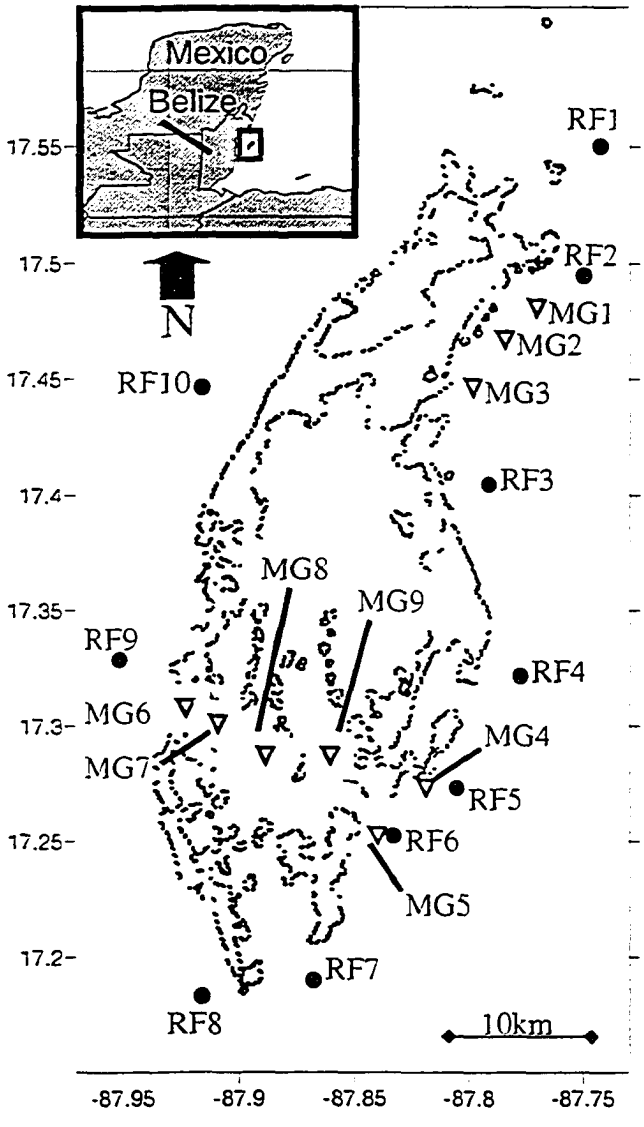
Table 6.1: Elements included in statistical analyses that were determined from the coefficient of variation (CV) of NIST samples being < 10%. and > 50% of otolith samples greater than detection limit. Average and standard deviation (SD) provided for detection limit by sample ablation and overall concentration (parts per million). Average CV is the mean of CV of 15 different sets of otolith ablations.

Isotope measured (atomic mass)	Average. CV (SD)	% of samples > detection limit	Average detection limit by sample (SD)	Average concentration (SD)
Li (7)	5.27 (0.21)	89	0.02 (0.01)	0.12 (0.03)
Mg (25)	1.84 (0.08)	99	0.96 (0.19)	14.48 (6.21)
Cu (65)	4.24 (0.26)	60	0.31 (0.23)	1.63 (1.53)
Zn (66)	3.80 (0.17)	76	0.17 (0.17)	1.67 (1.93)
Rb (85)	3.28 (0.13)	69	0.03 (0.01)	0.12 (0.03)
Sr (86)	1.32 (0.05)	99	1.10 (0.26)	3272 (534)
Sn (120)	3.54 (0.12)	99	0.02 (0.00)	0.77 (0.63)
Ba (138)	1.79 (0.08)	61	0.01 (0.00)	4.44 (2.12)
Pb (208)	4.00 (0.18)	86	0.01 (0.03)	0.15 (0.20)

Figure 6.1: Geographic position (decimal degree) of mangrove and coral reef sites throughout Turneffe Atoll, Belize. Triangles and circles indicate mangrove (MG) and coral reef (RF) sites, respectively. Site sample sizes provided in adjacent table.



Site	*Sample Size*
MG1	12
MG2	9
MG3	10
MG4	5
MG5	7
MG6	9
MG7	4
MG8	4
MG9	5
RF1	8
RF2	9
RF3	10
RF4	13
RF5	9
RF6	9
RF7	10
RF8	9
RF9	10
RF10	10

Figure 6.2: Adjusted $\log_{10}$ transformed otolith elemental concentrations (ppm) of Li, Mg, Zn, and Rb per habitat and site. Mean and standard error are plotted. Statistical significance from nested MANCOVA is indicated (i.e., comparisons between habitats and among sites within habitats).

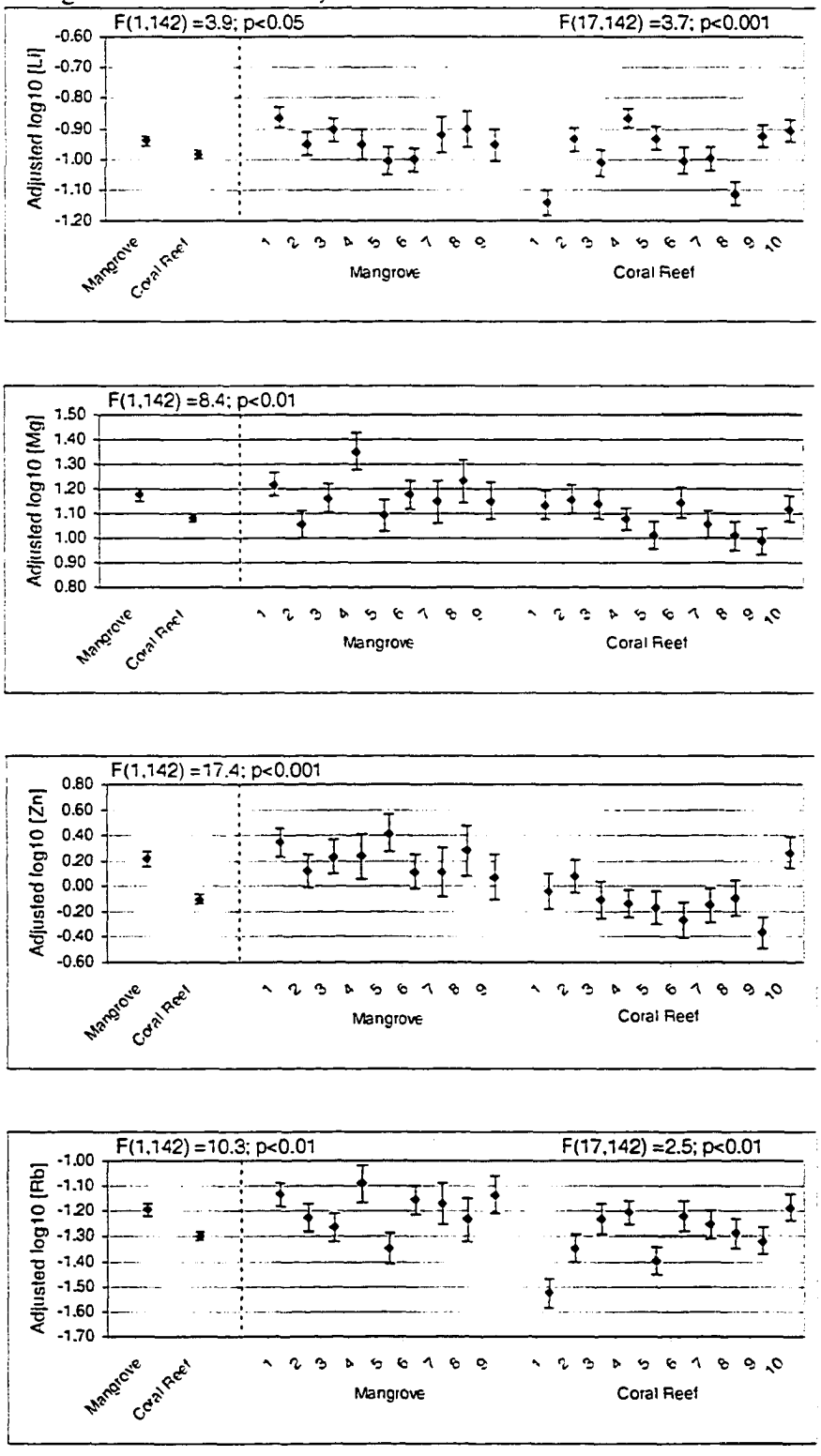
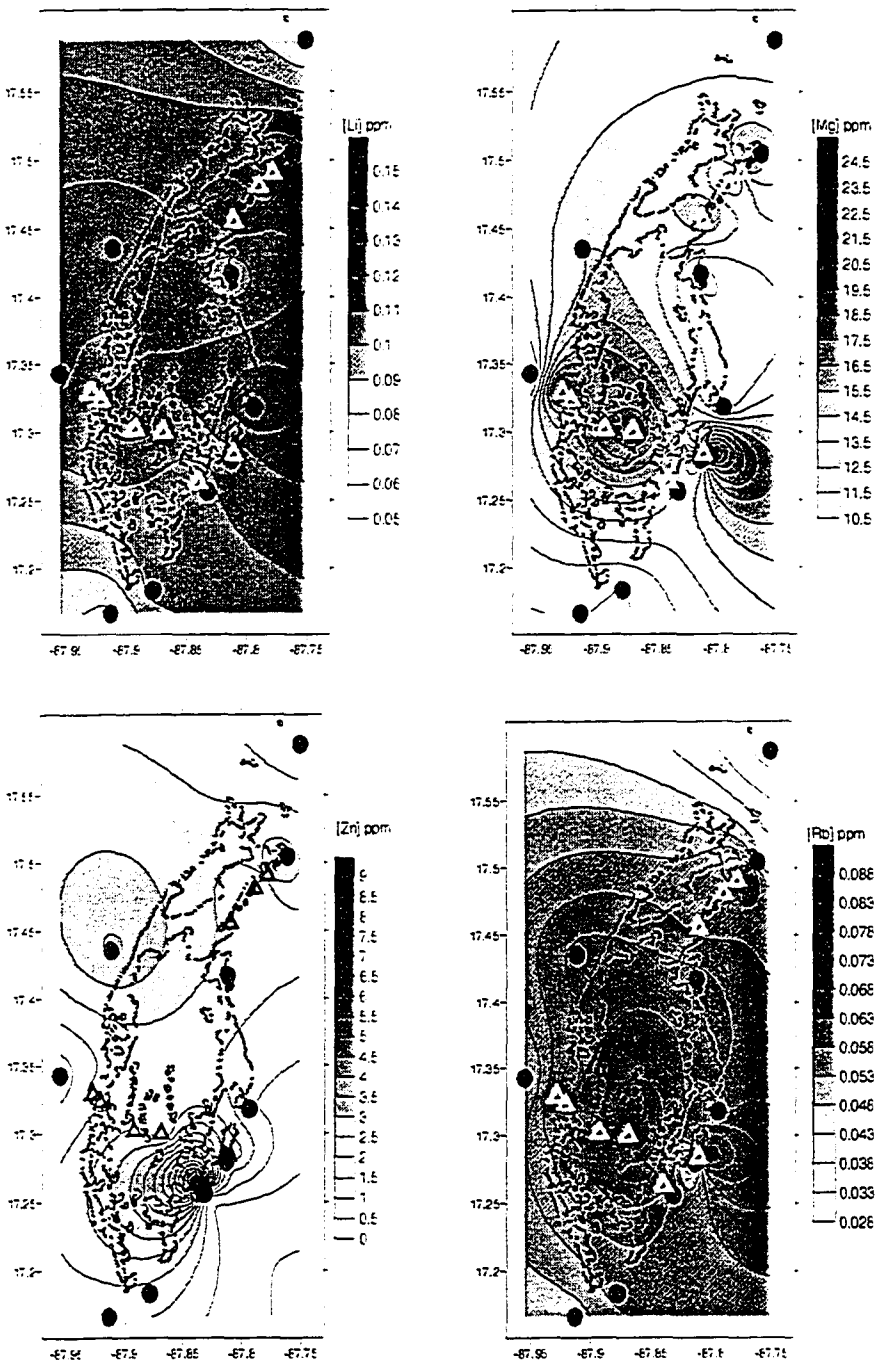


Figure 6.3: Spatial gradient of otolith elemental concentrations (ppm) of Li, Mg, Zn, and Rb throughout Turneffe Atoll, Belize. Contour plots are of untransformed concentrations (ppm). Triangles and circles indicate mangrove (MG) and coral reef (RF) sites, respectively.



Chapter 7: General Discussion

Introduction

The maintenance of a population is dependent upon the introduction of individuals (i.e., through birth and immigration) being equivalent to their removal (mortality and emigration). Any population may experience local extinction, yet through the dispersal of individuals among populations large-scale persistence can occur from the maintenance of populations with declining abundances, and the establishment of new populations by immigrants (Hanski 2001). An understanding of the dispersal path is therefore necessary in order to understand what shapes the distribution and abundance patterns for a particular species, and thus provide important information to aid in the effective conservation and management of species (particularly those that are of direct economic relevance).

For coral reef fish, aspects of their biology have hindered understanding where they originate. Specifically, reef fish movements have been difficult to track because of the inability to follow large numbers of small (< 5 mm) larvae that occupy the pelagic environment for days to weeks, and potentially travel great distances (10s to 100s of km; Cowen 2002). Making matters worse, these larvae are believed to experience high mortality, and therefore even if dispersing larvae could be followed most would die en route (Planes 2002). Since the direct observation of the movement of individuals through their entire life (from hatching, into pelagic larval phase, and then into a demersal habitat) is unlikely, methods are needed to help identify where fish came from. A common approach dating back to the 1600's is to tag individuals and thus allow their identification as they disperse (Guy et al. 1996). However, the quantification of the movement of larval and juvenile fish (as was the case for Section A and B, respectively, of this thesis) has several limitations that must be considered; the tag must not distort an individual's

natural behaviors or increase their mortality, it must be retained for the time frame in question (e.g., pelagic larval duration is approximately 30 days for *S. partitus*), be easily detectable, and it must be inexpensive and easy to administer (see reviews by Guy et al. 1996; Thorrold et al. 2002).

To investigate the dispersal of fish, several different tagging methods have been developed, which can be categorized as either artificial (organisms are tagged by the researcher) or natural tags (organisms are tagged through the natural variation in gene frequencies or chemical differences in the environment) (Thorrold et al. 2002). Artificial tags can be further classified to those tagging methods in which the researcher administers a mark either externally or internally. For example, external artificial tags, such as floy, spaghetti, and t-bar anchor, are highly visible on the external surface of the marked individual (e.g., Richardson 2000). In addition, some internal tags, such as visible implant fluorescent tags and coded wire tags, although inserted internally, are located just below tissue to allow direct visual observation of live fish (see review by Guy et al. 1996; Dorsey 2000). Unfortunately, due to their size, these tags are often inappropriate for investigating the dispersal of pelagic larvae (Thorrold et al. 2002), yet have been used to understand demersal stage movement of larger, older fish (see Guy et al. 1996). Fortunately, there are artificial tagging methods that have been used to assess movements of fish regardless of size or age. For example, a widely used technique for identifying where hatchery-produced fish originated uses temperature manipulations to form identifiable structural marks on their otoliths (reviewed by Volk et al. 1999). In addition, manipulations of the chemical composition of fish structures (e.g., bone, otolith, scales) have been successful at tagging fish regardless of size and age. For instance,

larval fish were labeled with tetracycline (see Jones et al. 1999), alizarin (see Fox et al. 2003), and radioactive elements such as ^{85}Sr (Vuorinen et al. 1998; Sutela et al. 2004), (see reviews by Geffen 1992; Thorrold et al. 2002). Even though these artificial tagging techniques have proven successful, they all suffer the same limitation: the substantial effort and cost required to collect individuals, mark, release, and the eventual recapture of some of them. Alternative approaches, such as those that utilize natural tags, have been developed that take advantage of the natural variability in gene frequency and elemental concentrations to mark fish, and thus eliminate the need for the labor intensive collection and tagging of fish.

With the development of genetic and elemental technologies (such as gel electrophoresis and inductively coupled plasma mass spectrometry, respectively), naturally occurring information found within various anatomical structures of fish has been used to delineate populations and assess the dispersal of individuals. For example, Taylor and Hellberg (2003), examined *Elacatinus evelynae* (Cleaner goby), a Caribbean coral reef species, and with the use of mitochondrial cytochrome b, found that despite a pelagic duration of 21 days, and thus the potential for long dispersal, populations showed strong genetic differentiation. However, the degree to which a genetic marker (allozymes, mitochondrial DNA, microsatellites, and nuclear sequences) is under selective pressure (e.g., allozymes and mitochondrial DNA are generally under selection, while microsatellites are neutral markers; Planes 2002) will influence inferences of population homogeneity, and thus determine the marker's applicability to addressing questions of connectivity (Hellburg et al. 2002). Alternatively, environmentally derived markers, such as geochemical signatures found in calcified structures like bone (see

Pollard et al. 1999), scales (see Wells et al. 2000 a and b, Wells et al. 2003 a and b), and otoliths (see reviews by Campana 1999; de Pontual and Geffen 2002, Thorrold et al. 2002), have also been used to investigate population discrimination and the movement of individuals (also see work using non-calcified structures such as eye lenses; see Dove and Kingsford 1998). *Although these calcified structures all grow continuously and record* chemical aspects of the environment, the otolith is unique in that it is metabolically inert (bones and scales have been shown to degrade during periods of stress; see Campana and Thorrold 2001; de Pontual and Geffen 2002; Wells et al. 2003a). Consequently, otoliths form permanent records of the elements sequestered onto their growing surface, and thus are preferentially used in chemical investigations because of the potential for retrospective analyses.

The successful application of otolith microchemistry as a natural tag to assess fish movement first requires sufficient geographic variability in otolith microchemistry at relevant spatial scales (Hamer et al. 2003). In each of the chapters of this thesis, significant spatial variability in otolith microchemistry was detected among collections of *Stegastes partitus* (Chapters 2, 3, and 4) and *Haemulon flavolineatum* (Chapters 5 and 6). To assess dispersal of individuals, this spatial variability in otolith microchemistry was used as a chemical map to which the chemistry of an otolith at an earlier time was compared. More specifically, comparisons were made between the chemical map and the otolith chemistry corresponding to the time *S. partitus* were hatched (i.e., otolith core was used to asses pelagic stage connectivity, Section A) or to the time *H. flavolineatum* were juveniles (i.e., after the settlement mark to assess demersal stage connectivity, Section B). Thus, any similarity in chemistry between a site from the chemical map and the otolith

chemistry of the hatching or juvenile period suggests a likely origin of a larva or site occupied by a juvenile, respectively, prior to dispersal. For example, in Chapter 2 the otolith edge chemistry of *S. partitus* collected throughout Tumeffe Atoll was used as the chemical map against which the otolith core chemistry of individuals from a subsequent collection were compared. The results of this analysis estimated that 6 to 35% of the individuals sampled (48 to 53 individuals) had pelagic larval dispersal that originated and finished within Tumeffe Atoll. In Chapter 5, the otolith edge chemistry of *H. flavolineatum* collected from mangroves and coral reefs represented the chemical map, which was later compared to the otolith chemistry corresponding to the juvenile life history stage of individuals taken from a coral reef. Results of this analysis indicated that 36% of 39 individuals taken from the reef had a chemical signature more representative of one of the mangrove sites, thus suggesting that they had occupied a mangrove and later migrated out to a reef. Although these examples highlight the success of the application of otolith microchemistry in order to understand dispersal, there are limitations of the technique that need to be addressed.

The use of otolith microchemistry to discriminate populations and assess connectivity (i.e., the demographic link maintained between populations of a species due to the movement of individuals; Mora and Sale 2002) relies upon complex biological processes that influence the deposition of trace elements, as well as equipment that requires expertise to operate. If otolith microchemistry is to be an effective tool in fish ecology, work needs to focus on ways to address issues of elemental uptake as well as improve the accuracy and precision of the detection of otolith microchemical concentrations. Consequently, the remainder of this discussion focuses on aspects of

otolith microchemistry that I feel warrant attention, and which deal with the biological nature of otoliths as well as the technical nature of their quantification. Specifically, I will address the time it takes for the otolith chemistry to be in equilibrium with the environment, crystal polymorphism, embryonic development, reference material, and otoliths as three-dimensional structures.

Biological processes that influence otolith microchemistry

Time to equilibrium

One approach to assess the dispersal of individuals requires that a chemical map is derived based on otolith edge chemistry, under the assumption that the edge, and thus its chemistry, corresponds to a point in the life of the fish just prior to its collection (this assumption is used throughout the thesis; Chapters 2 to 6). But to what degree does the chemistry at the otolith edge actually reflect where and when a fish was collected? In other words, is there a lag between when the ions of an element are in the environment and when they are deposited onto the otolith?

Although some information is available about the mechanism of trace element incorporation from the endolymph onto the otolith surface, very little is understood about the path prior to this (i.e., from the external environment to the endolymph) and the time that it takes. To address issues of initial uptake of elements, several studies have investigated whether trace elements found within otoliths are the product of dietary or environmental uptake (i.e., respiration and/or osmoregulation). Many of these studies have indicated that otolith elemental concentrations reflect that of the environment (e.g., Sr- Farrell and Campana 1996; Gallahar and Kingsford 1996; Bath et al. 2000; Milton

and Chenery 2001; Kennedy et al. 2002; Elsdon and Gillanders 2003; Ba- Bath et al. 2000; Milton and Chenery 2001; Elsdon and Gillanders 2003; Li- Milton and Chenery 2001; Pb- Geffen et al. 1998; and see Wells et al. 2000a and 2003a for evidence of trace element relationships between scales and water), but few studies provide information as to how long it takes for a change in the environment to be detected in the otolith (but see Gallahar and Kingsford 1996, Milton and Chenery 2001, and Elsdon and Gillanders 2005 below). Following the initial uptake of an ion into the internal environment of a fish, there are a series of filters that an ion must pass before being deposited onto the growing otolith, any of which can decouple the environmental concentrations from that of the otolith.

Elemental filtration begins at the gills during respiration and/or at the intestine during osmoregulation (marine fish swallow water to regulate their salinity). Those ions that make it into the blood will encounter another filter, this time between the blood and endolymph, whereby the concentrations of most major ions (e.g., P, Cu, S, and Ca) will be depleted because of physiological requirements (Kalish 1991; Campana 1999; Thorrold and Hare 2002). The final level of elemental filtration occurs between the endolymph and the growing surface of the otolith, such that elements are included if of a certain charge and/or size (see introduction for more details). Because filters are not suspected to noticeably impede the transfer of ions of most trace elements (e.g., Mn, Fe, Zn, Sr, Ba, and Pb) (Campana 1999) scientists utilize their concentrations to identify the elemental signature of the environment that a fish occupied. However, little information is available about the rates at which ions of these elements are transported into the otolith. Rojas-Beltran et al. (1995), reported that otoliths of *Coregonus lavaretus* (Common

whitefish) fry showed signs of a fluorescent mark after being immersed in oxytetracycline for as little as 1 minute, while other studies using different species at various life history stages, required several hours to ensure adequate uptake in the otolith to produce a visible tag (see Table 1 in Thorrold et al. 2002). Although most of these studies were concerned with the uptake of compounds into the otolith and not necessarily when the first signs of sufficient uptake appear, it still suggests that the rate of elemental uptake varies among species as well as developmental stage. In terms of the uptake of ions into otoliths, Milton and Chenery (2001), noted considerable variation in the rate of uptake among individuals of *Lates calcifer* (Barramundi), such that the concentrations varied for approximately two weeks before stabilizing. Specifically, concentrations of Sr and Ba from otoliths of fish transferred from freshwater to seawater revealed almost immediate changes, yet it took at least a week for them to stabilize. Similarly, Gallahar and Kingsford (1996) reported a lag in otolith Sr concentrations of *Girella elevata* (Black drummer), that varied among individuals from 5 to 15 days, when individuals were exposed to elevated concentrations of Sr. Finally, Elsdon and Gillanders (2005), observed a 20 day lag in otolith Sr concentration in *Acanthopagrus butcheri* (Black bream) regardless of the level of exposure (i.e., 2 or 4 times greater than ambient concentration).

If the temporal lag in otolith chemistry varies among individuals but is of the same range as was noted above (i.e., 5 to 20 days), then discriminating populations and assessing dispersal may be difficult since the otolith edge chemistry would represent the environmental signal from days to weeks prior to collection. Consequently, the site-specific chemical signals may become degraded, resulting in greater overlap in the map

of otolith chemistry, thus limiting the ability to discriminate populations as well as assess the movement of individuals. Although only a relatively small number of studies have reported difficulty in discriminating collections of individuals through space (e.g., Chapter 6 and Gillanders et al. 2001), it will be difficult to discern whether the lack of spatial heterogeneity in otolith chemistry is due to an environmental effect (e.g., lack of fresh water inputs that would alter salinity, temperature etc.), the movement of fish distorting spatial environmental signatures (see Gillanders et al. 2001 and Chapter 6), variability among individuals in the time it takes the otolith chemistry to reflect the environment, or any combination of these. In addition, whether the variability in the rate of uptake of ions is constant regardless of developmental stage (and thus different physiologies, see Campana and Gagne 1995; Chapter 4, and below) is a concern especially when comparisons are made between otolith core (representing embryonic stage) and edge (representing juvenile or adult stage) chemistry (see Chapter 2 and 5). Clearly, work needs to be done to identify the rate at which material is deposited into the otolith.

Development-related variability in otolith microchemistry

To assess pelagic larval connectivity using natural tagging methods, one approach is to compare spatially variable post-settlement otolith chemistry (i.e., edge chemistry corresponding to the known site of collection, but see above for discussion on this assumption) to otolith core chemistry (corresponding to the unknown site of origin). Specifically, post-settlement individuals are collected throughout the study location and their otoliths chemically analyzed at their edge, thus providing a time-specific chemical map of the study location. This chemical map is then compared to the otolith core

chemistry of fish from a subsequent collection of individuals, which hatched during the collection used to derive the chemical map. Any similarity in elemental concentrations between a site from the chemical map and the core chemistry of an individual likely indicates the hatching location of a given fish. However, an assumption of this approach is that the deposition of trace elements onto the growing otolith is consistent regardless of age or developmental stage, and thus otolith chemistry from hatching individuals (i.e., core chemistry) should be equivalent to that of juveniles and adults (i.e., edge chemistry) from the same place and time. Work by Fowler et al. (1995) and de Pontual et al. (2003) on *Micropogonias undulatus* (Atlantic croaker) and *Solea solea* (Sole), respectively, highlighted that both ontogenetic and environmental influences were involved in determining the early stage otolith chemistry. In addition, both Toole et al. (1993) and Otake et al. (1994) showed patterns in the concentration of Sr that were consistent with hatching and metamorphosis of *Microstomus pacificus* (Dover sole) and *Anguilla japonica* (Japanese eel), respectively.

In Chapter 4, I reported on an in situ experiment to assess ontogenetic variability in otolith microchemistry, in which the otoliths of *S. parvus* embryos were microchemically analyzed, and compared to that of juveniles from the same place and time. Our analyses indicated that the concentrations of trace elements were substantially higher in otoliths of embryos than juveniles. Similarly, Brophy et al. (2004) observed elevated concentrations in the cores of juvenile *Clupea harengus* (Atlantic herring) and *Sprattus sprattus* (Sprat), and suggested that the elevated concentrations were not the result of water or sediment contributions, but instead speculated that it might be linked to aspects of variation in otolith crystal structure and/or embryonic development.

Crystal polymporphism

In Chapter 4, I speculated that the type of otolith crystal structure, commonly aragonite, but sometimes vaterite or calcite, could have influenced the elevated concentrations in otoliths of embryos relative to that of juveniles. Unfortunately, there is limited information on whether crystal structure influences the uptake of ions and whether crystal structure varies ontogenetically. Depleted Sr concentrations have been observed in areas of vaterite from otoliths of *Stenodus leucichthys* (Inconnu) and *Oncorhynchus tshawytscha* (Chinook salmon) (Brown and Severin 1999 and Gauldie 1996, respectively). In addition, calcite in the shell of albalone has been shown to have higher concentrations of some elements relative to aragonite portions (Bettiol et al. 1999), and since calcite portions have been observed within otoliths of *Pollachius virens* (Pollock) (Strong et al. 1986), it is possible that elevated concentrations (as observed by Brophy et al. 2004, and in Chapter 4 of this thesis) may occur in calcite regions of otoliths. If the assessment of pelagic larval dispersal using otolith microchemistry is to continue to be investigated by comparing otolith edge and core elemental concentrations, then more work is needed to ensure that it is an effective approach. Therefore, the influence of calcium carbonate polymorphs on the deposition of trace elements must be reconciled.

Embryonic development

In terms of embryonic development and its role in elevated elemental concentrations, Volk et al. (2000) examined the maternal effects on otolith chemistry by crossing adult *Oncorhynchus tshawytscha*, *O. kisutch*, or *O. nerka* (Chinnok, Coho, and Sockeye salmon, respectively) that were captive within freshwater systems their entire

life, to those that were wild anadromous. Results of this study indicated that where the mother lived, and not necessarily the environment in which the eggs were spawned, influenced the concentrations of Sr in the offspring. In fact, from the time the eggs were spawned until hatching, Volk et al. (2000), regarded them as a closed system in which the source of trace elements in the otolith was from the yolk sac. Thus, influences from the maternally-associated environment (in this case freshwater or saltwater environment) are likely to have been transferred to the offspring via its yolk sac. In *S. partitus*, embryonic and early larval development is promoted by yolk sac nutrients that are depleted two days after hatching (Wilson and Meekan 2002). Since the embryos analyzed in Chapter 4 were collected hours prior to hatching, it is likely that the yolk sac was distorting the environmental signal in their otoliths. Therefore, if the otolith core is targeted for chemical analysis it is possible that the chemical signal will be a combination of yolk sac and environmental signal, thus complicating our ability to assign a site from which an individual originated and reducing the accuracy of this assignment (see Chapter 2).

The elevated elemental concentrations observed in otoliths of *S. partitus* embryos may also have resulted from Ca-binding proteins in the blood (Kalish 1989 and 1991) and their variation with age or ontogeny. According to Kalish (1989 and 1991), there is a positive correlation between Ca in the blood to that in the endolymph (i.e., the fluid surrounding each otolith), but because of Ca-binding proteins in the blood there is a decrease in free Ca that is available to enter the endolymph (likely through paracellular transport, Kalish 1991). Therefore, if Ba concentrations, for example, remained constant in the blood but free Ca decreased, the result would be a relative increase in Ba levels in the endolymph and concomitant increase in Ba in the otolith (Kalish 1989 and 1991).

Since Kalish (1989) reported that the number and type of proteins in the blood influenced age-related variation in otolith Sr concentration, it is possible that during embryonic development the proportion of Ca-binding proteins limits the amount of Ca entering the endolymph and thus the otolith, relative to that during the juvenile life stage.

Finally, the elevated concentrations in embryo otoliths relative to that of juveniles may result from a large proportion of protein relative to calcium carbonate (in the form of aragonite) in the embryo otolith of *S. partitus* (three days post-fertilization). Specifically, at approximately 2 days (50 hours) post-fertilization (corresponding to a 16 μm diameter otolith), Ca was first detected in the edge of the otolith (corresponding to a 1 μm width) in *Danio rerioi* (Zebrafish) (Pisam et al. 2002). Consequently, microchemical analyses of embryos three days post-fertilization would consist largely of a protein nucleus with an exterior portion containing Ca. Two problems arise from this possibility. First, since little is known about the trace elemental composition of the otolith protein portions, it is possible that our elemental concentrations are related to the protein nucleus containing high concentrations of trace elements (possibly derived from the yolk sac, see above). Secondly, in the data acquisition process, Ca is used as the internal standard, since it comprises 90-95% of the otolith (Pisam et al. 2002). However, in embryonic otoliths, the concentration of Ca may be less significant, and thus using it as an internal standard will lead to inaccurate estimates of trace elemental concentrations.

Overall, we reported that the chemical signal varied with development stage and speculated as to the cause of this variability: crystal structure (i.e., aragonite versus vaterite or calcite) and/or embryonic development (i.e., yolk sac contribution, Ca-binding proteins, and otolith protein content). Consequently, analyses of connectivity that are

based on trace elemental concentrations of otolith cores and edges should be cautiously interpreted (see Chapter 2) until a better understanding is obtained of the deposition of ions onto the otolith during embryonic development and later in life.

Instrument-related factors

Since the late 1970s, there has been rapid growth in research related to the elemental composition of otoliths (Figure 1.1), and thus a concomitant increase in the number of labs with instrumentation, such as inductively coupled plasma mass spectrometry, for quantifying geochemical signatures. Although the use of such equipment is beyond the scope of this chapter (but see Jeffries 2001; de Pontual and Geffen 2002 for more information), there are aspects of its operation that are briefly discussed because of the profound impact they have on the measurement of elemental concentrations. For instance, to ensure accurate and precise microchemical analyses, certified reference materials (CRM) are used, yet there is little consistency in the type of CRM used among labs and thus it is impossible to compare studies (Yoshinaga et al. 2000). Furthermore, with the development of analytical techniques that permit sampling at specific locations on the otolith, such as laser ablation and micromilling, there is a need to consider the three dimensional nature of otoliths and the amount of material analyzed.

Reference materials

In research that involves chemical analysis, quality assurance is essential (Yoshinga et al. 2000). This is typically accomplished through the use of CRM (such as those from the National Institute of Standards and Technology, NIST) that often consists of a homogenous glass that is spiked with a range of elements of known concentrations.

NIST CRMs are typically used in earth science applications since its behavior their similar to that of materials commonly analyzed (Jeffries 2001). However, for applications outside of earth sciences, alternative CRMs are preferred that are more similar to the matrix (i.e., the type of material and its structure) under analysis (Jeffries 2001; de Pontual and Geffen 2002). For instance, in otolith microchemical analyses, the CRM should account for the protein component as well as the calcium carbonate component (de Pontual and Geffen 2002). For example, Yoshinga et al. (2000) reported on the production and analysis of a CRM that was prepared from sagittal otoliths of *Lutjanus sebae* (Red emperor), while Campana et al. (1997) and Thorrold et al. (1997) prepared a CRM that was used in inter-laboratory comparisons. Yet, apart from these afore mentioned studies, there has been little uniformity among ICP-MS labs regarding the use of specific CRMs.

A inspection of microchemical studies unfortunately indicates that the type of CRM used varies substantially, yet to facilitate comparisons among studies, there is need for the production and standardized use of a CRM that is applicable to all otolith microchemical analyses (Yoshinga et al. 1997). For example, in Chapter 4 of this thesis, I reported on elevated elemental concentrations in the otoliths of *S. parvitus* embryos relative to that of juvenile otoliths, and sought an explanation for this pattern in other studies (e.g., Michibata and Hori 1979; Brophy et al. 2004; Patterson et al. 2004). Yet, because of differences in analytical settings and the CRM used among studies, direct comparisons of concentrations were impossible. Because of this inability of inter-laboratory comparisons, interesting patterns in otolith microchemistry are likely being overlooked. Although equipment (laser and ICP-MS), tuning and calibration, and

conditions of operation vary from lab to lab, standard CRM will reduce confounding effects that would otherwise prevent meta-analyses of otolith microchemical studies. Therefore, a more unified analytical approach needs to be adopted in order to ensure accurate and precise investigations across labs.

Otoliths as three-dimensional structures

Otoliths are three-dimensional structures that begin, more or less, as circular discs (Pisam et al. 2002; and see Chapter 4), but eventually become oblong because of uneven growth. Although this microstructure is irrelevant for bulk analyses of otoliths (i.e., the complete dissolution of an otolith) surface analyses that utilize laser or micromilling equipment to sample specific portions of the otolith (corresponding to particular times in the individuals' life) must take into account this complex configuration. For example, if the laser-sampling radius was 20 μm in diameter and a series of spot samples were taken across the surface of a transverse section of a sagittal otolith, then each spot will have sampled a different length of time in the life of a fish because of uneven increment deposition. Furthermore, Jones and Chen (2003) reported that retrospective analyses of the core region of juvenile otoliths would likely encompass a region larger than that intended. Specifically, they noted that a maximum crater depth of 80 μm was sampled even though a10 μm laser diameter was used. Consequently, the depth of the analysis would incorporate otolith material not of interest to the researcher. Overall, this means that the sampling of otoliths requires care and a consistent protocol to reduce confounding affects that may occur from the addition of unwanted material.

The over-sampling of otolith material (as noted by Jones and Chen 2003) was apparent when comparing the otolith elemental concentrations of *S. parilus* embryos (three days post-fertilization: Chapter 4) to the retrospectively determined concentrations from the cores of juvenile otoliths (Chapter 2). Although the same laser sampling settings were used, the elemental concentrations in embryo otoliths were substantially elevated relative to the cores of juvenile otoliths. This dichotomy in elemental concentrations could be explained in terms of the differential sampling of material between embryo otoliths and juvenile cores. Specifically, the chemical analysis of embryo otoliths (average diameter of 18μm) was limited by their size and thus the elemental concentrations consisted solely of this embryonic period. However, even though the retrospective analysis of juvenile otoliths targeted the core, additional layers that were deposited after hatching were likely sampled (as was noted by Jones and Chen 2003), thus distorting the elemental concentrations relative to that from the embryo otoliths. Regardless of the portion of the otolith that is targeted, there will likely be the removal of additional material (potentially representing several days in the fish's life) depending on sampling radius, laser power, and speed of traverse. Therefore, an awareness of the three dimensional sampling is suggested to limit confounding affects that could alter our ability to discriminate populations or assess the movement of individuals.

Conclusion

This thesis represents the culmination of separate investigations of pelagic and demersal life history stages that were unified through the use of otolith microchemistry to

assess population structure and the degree of connectivity among them. The usefulness of otolith microchemistry is apparent when the contents of this thesis are amassed. Specifically, I have reported on the ability to discriminate collections of *S. partitus* and *H. flavolineatum* through space and time, the relative degree to which larval populations are connected, and the extent to which adult reef fish utilized an alternative habitat during their development. In addition, I presented a method (chemical analysis of lapillus and sagittal otoliths) that will refine and improve our ability to discriminate populations, while also highlighting the need for better understanding of the processes involved in the deposition of trace elements throughout the life history of an individual (time to equilibrium, crystal polymorphism, embryonic development, and the structure of otoliths). Clearly, the study of the distribution and abundance of fish will continue to benefit from the use of otolith microchemistry.

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